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THE DESIGN, SYNTHESIS AND SCREENING OF POTENTIAL PYRIDINIUM OXIME PRODRUGS

Final Report

Ronald T. Borchardt

September 30, 1985

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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University of Kansas
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In an attempt to improve the delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE) to the central nervous system (CNS), structural analogs and prodrugs of N-methylpyridinium 2-carbaldoxime (2-PAM) have been synthesized. The potential prodrugs are dihydropyridinium oximes (pro-2-PAM's) or tetrahydropyridinium oximes, which possess electron-withdrawing substituents in the 3- or 5-position. As precursors to these

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prodrugs, we have synthesized and characterized a series of 5-substituted-2-PAMs (I, Br, Cl, CH_3, CN, $CONH_2$-substituted) and a series of 3-substituted PAMs (I, Br, Cl, CH_3-substituted). These analogs were tested in vitro for their ability to reactivate diisopropylfluorophosphate (DFP)-inactivated eel AChE. The following order of potency was observed: 3-chloro-2-PAM > 3-bromo-2-PAM > 3-methyl-2-PAM ≥ 3-iodo-2-PAM > 5-methyl-2-PAM ≥ 2-PAM ≥ 5-chloro-2-PAM > 5-carboxamido-2-PAM ≥ 5-bromo-2-PAM > 5-cyano-2-PAM ≥ 5-iodo-2-PAM. When tested for their ability to protect mice from a challenge dose (2 X LD_{50}) of DFP, the following order of potency was observed: 5-iodo-2-PAM >> 3-iodo-2-PAM ≥ 2-PAM > 3-bromo-2-PAM = 5-bromo-2-PAM > 5-carboxamido-2-PAM > 5-methyl-2-PAM > 3-methyl-2-PAM = 3-chloro-2-PAM = 5-chloro-2-PAM >> 5-cyano-2-PAM. Many of these analogs have also been tested by the Walter Reed Army Institute of Research (WRAIR) for the ability to protect mice from a challenge dose of soman (2 X LD_{50}). 5-Iodo-2-PAM and 5-bromo-2-PAM were more effective than 2-PAM when administered with atropine. In the same test system, 3-chloro-2-PAM and 3-bromo-2-PAM were less active than 2-PAM, whereas 3-iodo-2-PAM, 5-cyano-2-PAM and 5-carboxamido-2-PAM were inactive.

Pro-2-PAM has been synthesized and its biological properties studied. However, attempts to prepare other 3- or 5-substituted dihydropyridinium oximes (pro-2-PAMs) have not been successful. Several tetrahydropyridinium oximes (CN, SCN and Br adducts of pro-2-PAM) have been synthesized and characterized. Conversions from the tetrahydropyridinium oximes to 2-PAM were measured under physiological conditions (pH 6.5-8.5) and only with the CN adduct was there appreciable conversion to 2-PAM.

To aid in the in vitro biological evaluation of the potential regenerators of AChE, a new screening assay was developed, employing immobilized AChE (eel, human RBC). The AChE activity was continuously monitored spectrophotometrically in a closed loop fashion, using acetylthiocholine and dithiobis (nitrobenzoic acid). The assay allowed for independent inactivation and reactivation of AChE, followed by the determination of regenerated AChE activity.

To aid in the in vivo biological evaluation of potential regenerators of AChE, a simple and reliable high performance liquid chromatography (HPLC) assay for pyridinium oximes (e.g., 2-PAM) in biological tissue was developed. The assay is sufficiently sensitive to allow for detection of 2-PAM and the dihydropyridinium prodrug (pro-2-PAM) in brain and other biological tissues (e.g., kidney) and fluids (e.g., blood).

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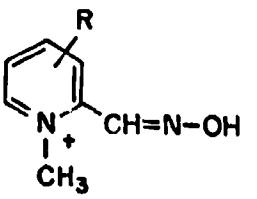
Summary

In an attempt to improve the delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE) to the central nervous system (CNS), structural analogs (I) and potential prodrugs (II, III) of N-methylpyridinium 2-carbaldoxime (2-PAM) have been synthesized. The potential prodrugs are dihydropyridinium oximes (II, pro-2-PAM's) or tetrahydropyridinium oximes (III), which possess electron-withdrawing substituents in the 3- or 5-position. As precursors to these prodrugs, we have synthesized and characterized a series of 5-substituted-2-PAM's (I, Br, Cl, CH_3, CN, $CONH_2$-substituted) and a series of 3-substituted-2-PAM's (I, Br, Cl, CH_3-substituted). These analogs were tested *in vitro* for their ability to reactivate diisopropylfluorophosphate (DFP)-inactivated AChE, *in vivo* for their ability to protect mice from a challenge dose (2 X LD_{50}) of DFP, and *in vivo* for their ability to protect mice from a challenge dose (2 X LD_{50}) of soman.



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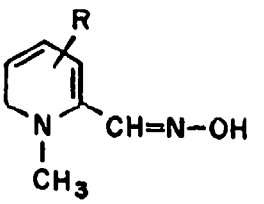

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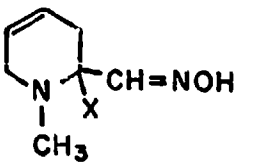

III

The following order of potency was observed in the *in vitro* DFP-inactivated AChE assay: 3-chloro-2-PAM > 3-bromo-2-PAM > 3-methyl-2-PAM ≥ 3-iodo-2-PAM > 5-methyl-2-PAM ≥ 2-PAM ≥ 5-chloro-2-PAM > 5-carboxamido-2-PAM ≥ 5-bromo-2-PAM > 5-cyano-2-PAM ≥ 5-iodo-2-PAM. When tested for their ability to protect mice from a challenge dose (2 X LD_{50}) of DFP, the following order of potency was observed: 5-iodo-2-PAM >> 3-iodo-2-PAM ≥ 2-PAM > 3-bromo-2-PAM = 5-bromo-2-PAM > 5-carboxamido-2-PAM > 5-methyl-2-PAM > 3-methyl-2-PAM = 3-chloro-2-PAM = 5-chloro-2-PAM >> 5-cyano-2-PAM. Many of these analogs have also been tested by the Walter Reed Army Institute of Research (WRAIR) for their ability to protect mice from a challenge dose of soman (2 X LD_{50}). 5-Iodo-2-PAM and 5-bromo-2-PAM were more effective than 2-PAM when administered with atropine. In the same test system 3-chloro-2-PAM and 3-bromo-2-PAM were less active than 2-PAM, whereas 3-iodo-2-PAM, 5-cyano-2-PAM and 5-carboxamido-2-PAM were inactive.

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To aid in the in vitro biological evaluation of the potential regenerators of AChE, a new screening assay was developed employing immobilized AChE (eel, human RBC). The AChE activity was continuously monitored spectrophotometrically in a closed loop fashion, using acetylthiocholine and dithiobis (nitrobenzoic acid). The assay allowed for independent inactivation and reactivation of AChE followed by the determination of regenerated AChE activity.

To aid in the in vivo biological evaluation of potential regenerators of AChE, a simple and reliable high performance liquid chromatography (HPLC) assay for pyridinium oximes (e.g., 2-PAM) in biological tissue was developed. The assay is sufficiently sensitive to allow for detection of 2-PAM and the dihydropyridinium prodrug (pro-2-PAM) in brain and other biological tissues (e.g., kidney) and fluids (e.g., blood).

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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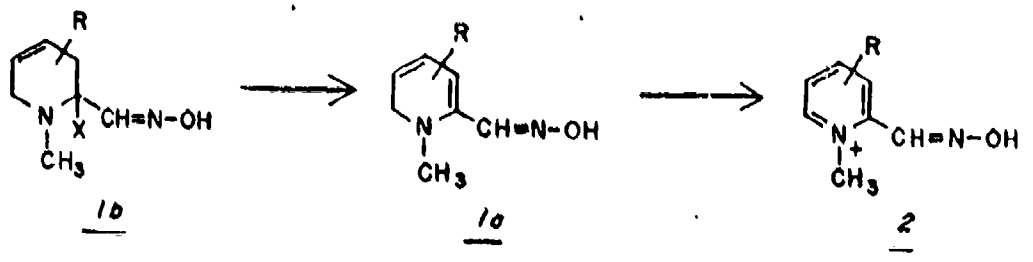
A. Problem

1. Design and Synthesis of Pro-PAM Agents

The overall objective of the project is to improve central nervous system (CNS) delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE). The specific chemical problem is to design and synthesize nonquaternary, lipophilic prodrugs of pyridinium oximes which can be easily transformed *in vivo* into active quaternary regenerators. The prodrug forms consist of dihydropyridinium oximes 1a or the tetrahydropyridinium oximes 1b, which possess electron withdrawing substituents in the 3- or 5-position. Prodrugs 1b require elimination and oxidation to generate the corresponding N-methylpyridinium 2-carbaldoximes (2-PAM's) (2), whereas prodrugs 1a require only oxidation. It is hoped that the electron withdrawing substituents will stabilize the prodrugs, resulting in a slower *in vivo* conversion and thereby improvement of biodistribution to the CNS.



Determination of the rate and efficiency of conversion from prodrugs to parent pyridinium oximes will be required for evaluation of therapeutic potential and reactivator structural refinement. The measurement of physical properties such as pKa, partition coefficient and water solubility will be required to predict membrane penetration, tissue disposition and ability to dephosphorylate AChE.

2. Biological Testing

All drug candidates will require evaluation as regenerators of organophosphate-inhibited cholinesterases. Effective *in vitro* and *in vivo* screens will be necessary to evaluate both the parent pyridinium oximes and prodrug forms. In addition, a simple and effective pyridinium oxime detection technique for tissue distribution and elimination studies will be required. These studies will be essential in evaluating regenerator efficacy and CNS penetration.

B. Background

1. Regenerators

Organophosphates as a class owe their toxicity to their ability to react covalently with the esteratic site of AChE. AChE is the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine. The phosphorylated enzyme can be reactivated by a variety of agents. There is, however, a competing "aging" process whereby the inactivating phosphoryl group either migrates to an adjacent amino acid residue[1] or is partially hydrolyzed.[2,3] Phosphorylated AChE which has undergone this "aging" process is not easily reactivated. Thus rapid reactivation of the poisoned enzyme in all affected tissues is highly desirable.

I. B. Wilson discovered 2-PAM (3), which is now one of the most widely used and therapeutically effective broad spectrum AChE regenerators.[4] Wilson postulated that the electrostatic attraction of the quaternary nitrogen helped orient the oxime moiety toward the phosphorylated esteratic site (Figure 1).[5,6]

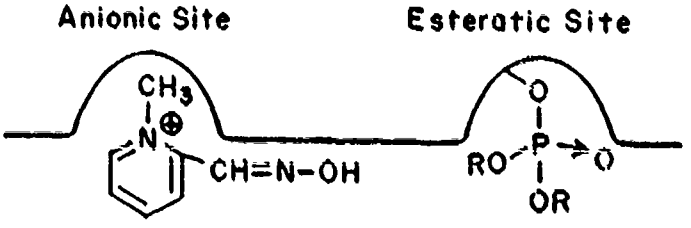


Figure 1. Proposed Active Site of AChE.

Since the discovery of 2-PAM, there have been a variety of other organic oximes which have been synthesized and screened for AChE regenerating activity (Figure 2). The charged bis-oximes trimedoxime (TMB-4) (4) and HI-6 (5) have both proven to be potent reactivators, but suffer from poor tissue penetration, short serum half-lives and toxicity problems.[7] Neutral oximes such as monoisonitrosoacetone (MINA) (6) and 5-hydroxyiminomethyl-3-phenyl-1,2,4-oxadiazole (7) have shown much less potent regenerator ability, but do possess better lipid solubility.[8-10]

Investigations into the structure activity relationships of substituted 2-PAM's have produced some interesting information. In general, electron-withdrawing substituents shift the pKa of the pyridinium oxime to values below the optimum range of 7.4-7.8, and electron donating substituents shift it higher.[4,11] Some 5-substituted 2-PAM's (Cl, CH_3) were approximately as effective as 2-PAM in whole animal survival studies, even though in vitro testing showed them to be less effective at regenerating deactivated AChE.[12]

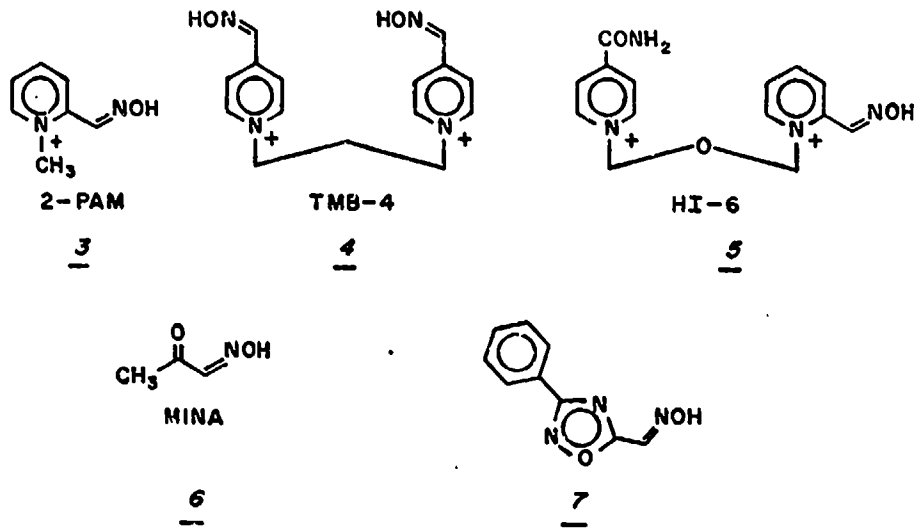


Figure 2. Regenerators of AChE.

These results indicate that the active site of AChE can tolerate minor structural changes in 2-PAM and, further, that desirable distribution characteristics might be incorporated into the molecule without significantly reducing its reactivating capabilities. In an attempt to increase the lipid solubility of 2-PAM, N-dodecyl-2-pyridinium carbaldoxime (2-PAD) was made. It possessed increased lipid solubility, but proved to be far less effective than 2-PAM at in vitro reactivation.[13]

Only trace amounts of 2-PAM can be detected in the CNS following i.v. injection.[1] Because of its high water solubility, the intact drug is rapidly eliminated from the body with an observed half-life in humans of less than 1 hour. Blood levels fall below the therapeutically effective range after 1-2 hours.[14,15] Significant brain levels of 2-PAM can be achieved only by intraventricular injections of the drug.[16,17]

Asphyxiation due to suppression of the central respiratory center is the ultimate cause of death in mammals exposed to anticholinesterase agents.[18,19] The lipid permeability of many organophosphates allows them to penetrate many body tissues, including the CNS, which are impermeable to a charged molecule like 2-PAM. Therefore, the need to regenerate AChE in the CNS presents an interesting drug delivery problem.

The delivery of 2-PAM to the highly lipid CNS was achieved with the pioneering work of Shek, Bodor and Higuchi.[20-23] The University of Kansas group synthesized a prodrug of 2-PAM. Working on the hypothesis that a tertiary amine would have little difficulty penetrating the CNS, they synthesized a partially reduced form of 2-PAM which was a latent quaternary amine. Figure 3 illustrates how they trapped the reduced form of 2-PAM as a cyanide addition product, 9, which upon careful

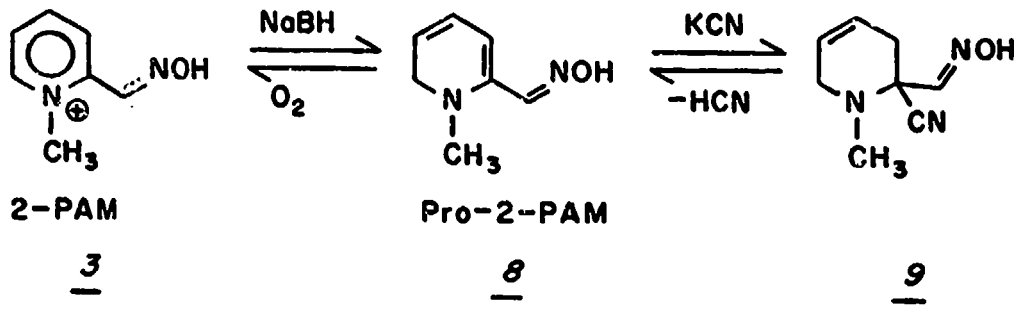


Figure 3. Synthesis of Pro-2-PAM.[20-23]

decomposition afforded pro-2-PAM (8). The pKa of 8 was determined to be 6.3, which was good for favorable physiological partitioning. They found that in vivo oxidation of 8 to 2-PAM (3), much like the NAD-NADH redox system, took place in approximately 1 minute.[22] This was sufficient time to allow the drug to cross the blood-brain barrier, producing a 13-fold increase in brain levels of 2-PAM.[23]

2. Biological Screening of Potential Regenerators

a. In Vitro Assays

Available in vitro screening techniques involve purified or partially purified soluble cholinesterase, which is exposed to an organophosphate and incubated with a regenerator, followed by the determination of enzyme hydrolytic activity. These assays generally involve the use of either acetylcholine or an analog which can be hydrolyzed by the enzyme. The resulting hydrolysis products can be measured colorimetrically, spectrally, and via pH change either directly or by CO_2 equilibria (both volumetric and radiometric).[8,9,24-27] By far the most convenient and sensitive method is that developed by Ellman et al.,[28] which utilizes acetylthiocholine as substrate and measures the formation of thiocholine by its reaction with a disulfide chromogen.[28]

b. In Vivo Assays

In vivo evaluation of oxime regenerators has employed mice, rats, guinea pigs and rabbits.[29-31] The determination of LD_{50} values for potential regenerators as well as ED_{50} values (vs. 2 X LD_{50} challenge of organophosphate) are well documented and provide reliable, effective and comparable data. The determination of protective ratios (PR), in which doses of regenerators raise the lethal dose of organophosphates required to produce death, provides useful information about potential therapeutics.

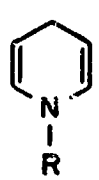
3. Biodistribution

Biodistribution patterns of pyridinium oximes have been determined, using three basic techniques; the administration of radiolabeled oximes followed by paper chromatographic identification is, to date, the most sensitive technique.[23,32] UV spectrophotometric quantitation of oximes in tissue extracts has also been employed; however, this does not provide positive identification of the measured species.[33] More recent developments involve the use of high performance liquid chromatography (HPLC) to identify and quantitate quaternary oximes in body tissues.[34]

C. Approach

1. Prodrug Design

The basic approach has been to reduce measurably the rate of oxidation of a dihydropyridinium oxime to its active parent quaternary form by stabilizing the dihydropyridinium structure with electron-withdrawing substituents. It is known from the literature that an electron-withdrawing group in the 3- or 5-position stabilizes the dihydropyridines 11 and 12, relative to 10, through electron delocalization.[35]



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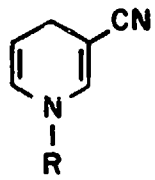

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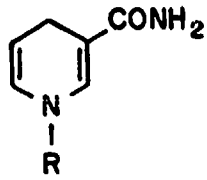

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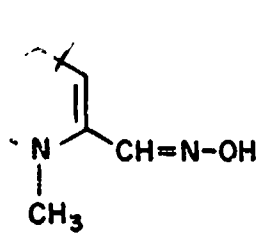
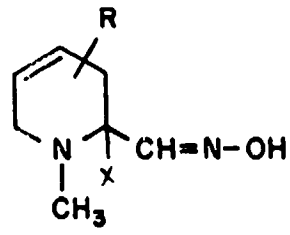
By synthesizing 3- and 5-substituted pro-2-PAM' 1a, it is hoped





R = I, Br, Cl, CN, $CONH_2$, CH_3

that the rate of conversion from prodrug to the active quaternary pyridinium oxime forms can be slowed, thus allowing more time for partitioning into the CNS compartment (Figure 4). There may very well be a tradeoff

between reduced reactivating ability of the substituted pyridinium oxime and increased tissue permeability of the prodrug form; however, this can be effectively determined only by in vivo testing.

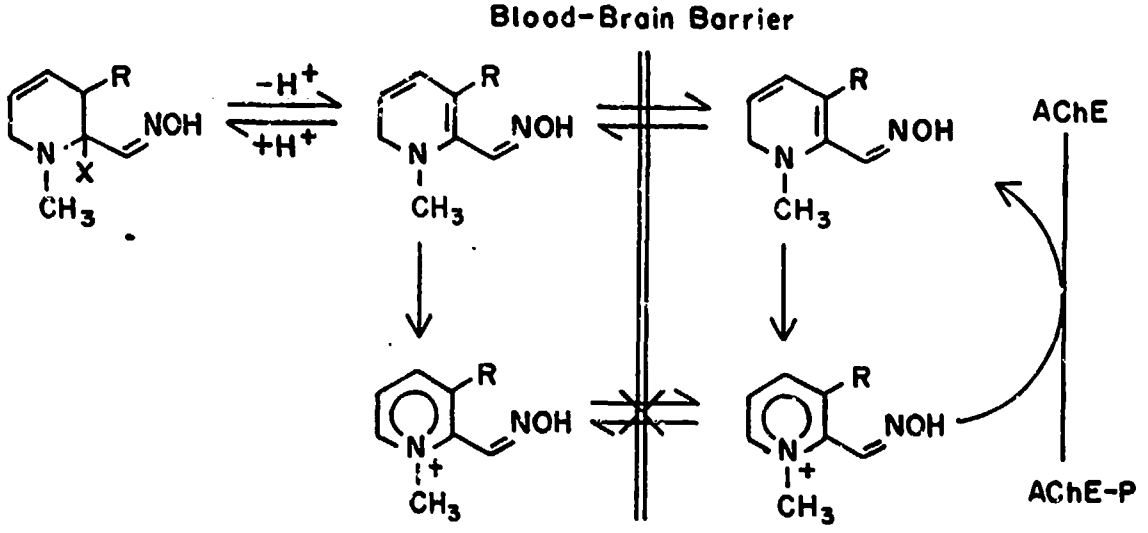


Figure 4. Prodrug Permeability of the Blood-Brain Barrier and Conversion to 2-PAM.

2. Biological Evaluations

a. In Vitro

We have focused our attention on continued development and validation of a reliable and efficient in vitro AChE assay as a preliminary screening technique for the potential new regenerators being synthesized in our laboratories. The basic Ellman technique appeared to be a viable assay which did not require any dedicated equipment other than a spectrophotometer.[28] The intent was to have a rapid and efficient assay to be used as a primary screen for the parent quaternary pyridinium oximes. In this manner the quaternary oximes which exhibit poor or negligible reactivation capabilities compared to 2-PAM could be identified and discarded. This would allow concentration of efforts toward synthesizing prodrug forms of the most active regenerators. In addition, many of the prodrugs are oxygen-sensitive, which necessitates that the assay be adaptable to anaerobic conditions. All of the above requirements (i.e., speed, reliability, sensitivity and adaptability to anaerobic conditions) led us to the Ellman technique to measure AChE activity and the use of an immobilized AChE.[36] We also intend to extend the in vitro screening assay to incorporate whole brain tissue and thus establish the relationship between purified enzyme behavior and that of enzyme in a biomatrix.

b. In Vivo

Working with mice, we have focused on the determination of LD_{50} and ED_{50} values (vs. 2 X LD_{50} DFP challenge) of new regenerators. In addition, work on protective ratios should provide information helpful in evaluating new regenerators.

c. Biodistribution

Our efforts have focused on the development of assays, which allow for quantitative determination of tissue uptake and elimination patterns of pyridinium oximes. The use of HPLC and UV detection allows positive identification and quantitation of quaternary oximes and their metabolites in tissues. The examination of silicon-based, reversed-phase columns eluted with aqueous/organic solvents containing ion-pairing reagents for mobility control as well as hydrocarbon resin-based, reversed-phase columns eluted with aqueous solutions at various pH's for mobility control allows identification of several general, universally applicable conditions for identification and quantitation of pyridinium oximes in all tissues, especially the CNS.

D. Results and Discussion

1. Chemistry

In this report we describe the successful synthesis of 3-methyl-2-PAM chloride (17a), 5-methyl-2-PAM iodide (16b), 3-bromo-2-PAM chloride (17c), 3-chloro-2-PAM chloride (17d), 5-chloro-2-PAM chloride (17e), 5-cyano-2-PAM chloride (17g), 5-carboxamido-2-PAM chloride (17k), 5-bromo-2-PAM chloride (17l), 3-iodo-2-PAM chloride (17m), 5-iodo-2-PAM chloride (17n), pro-2-PAM (8) and the cyanide addition product 9. We have also described our unsuccessful attempts to prepare 3-cyano-2-PAM iodide or tosylate (16f), 3-carboxamido-2-PAM iodide or tosylate (16j), the dihydropyridinium oximes 18g, 18k and 18n and the tetrahydropyridinium oxime adducts 1b (R=H, X=Br, SCN).

3-Methyl-2-PAM chloride (17a) and 5-methyl-2-PAM iodide (16b) were prepared from 2,3-lutidine (13a) and 2,5-lutidine (13b), respectively, using the steps outlined in Figure 5. Sufficient quantities of 3-methyl-2-PAM chloride (17a) were prepared and sent to the Walter Reed Army Institute of Research (WRAIR) for *in vitro* testing in soman-challenged mice.

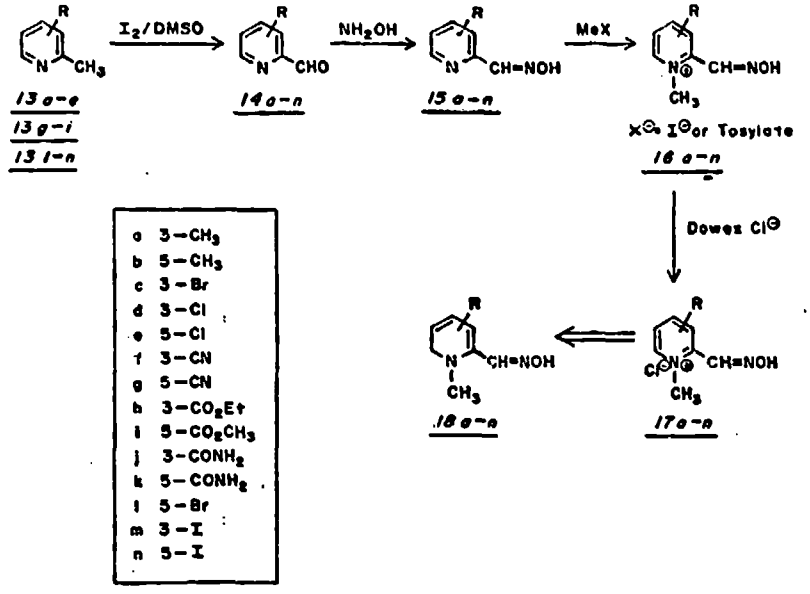


Figure 5. Strategies for Syntheses of 3- or 5-Substituted Pyridinium Oximes.

The synthesis of 3-bromo-2-PAM chloride (16c) and 5-bromo-2-PAM chloride (16l) was initiated with the bromination[37] of 2-picoline (19) to yield a mixture of 3-bromo-2-picoline (13c) and 5-bromo-2-picoline (13l). A mixture of the 3-bromo and 5-bromo-2-picolines 13c and 13l were oxidized (I_2/dimethyl sulfoxide [DMSO]) to 3-bromopicoline (14c) and 5-bromopicoline (14l). After chromatographic separation of the bromopicolines 14c and 14l, the individual isomers were converted to the 3-bromo and

5-bromo pyridinium oximes 15c and 151 (Scheme 5). The 5-bromo pyridine oxime 151 was quaternized with methyl iodide to yield 161 and then converted to the chloride salt 171, using ion exchange chromatography (Dowex Cl^- form). Methylation of the 3-bromo pyridine oxime 15c with methyl iodide was unsuccessful; however, 15c was successfully methylated with methyl tosylate to yield 16c as the tosylate salt. The oxime 16c was converted in good yield to 17c, using ion exchange chromatography (Dowex Cl^- form). Sufficient quantities of 3-bromo-2-PAM chloride (17c) and 5-bromo-2-PAM chloride (171) were prepared and sent to WRAIR for in vivo testing in soman-challenged mice.

Attempts to prepare 3-chloro-2-picoline (13d) and 5-chloro-2-picoline (13e) by reaction of the corresponding bromopicolines 13c and 131 with CuCl were unsuccessful. Attempted addition of methyl lithium to 3-chloropyridine also met with failure. However, a mixture of the chloropicolines 13d and 13e was prepared from a mixture of the aminopicolines 20 and 21, using the procedure of Talik et al.[38] (Figure 6). The mixture of the aminopicolines 20 and 21 was prepared from a mixture of the corresponding bromopicolines 13c and 131 according to the procedure of van der Does[37] (Figure 6). 3-Chloro-2-picoline (13d) was also prepared by reduction of 3,6-dichloro-2-picoline (25), using a modification of the procedure of Baumgarten et al.[39] 3,6-Dichloro-2-picoline (25) was prepared from 6-amino-2-picoline (22) as outlined in Figure 7.

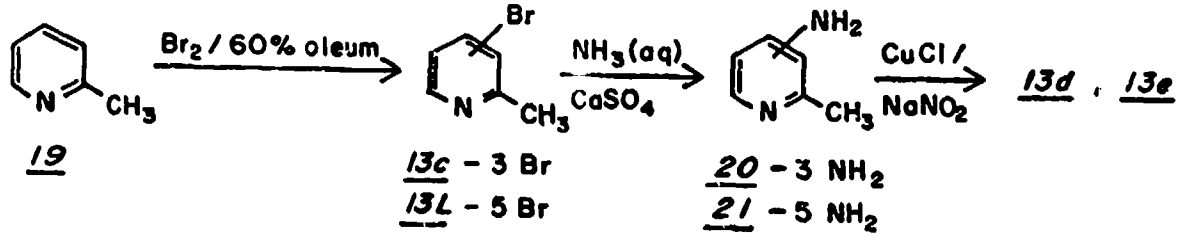


Figure 6. Synthesis of 3-Chloro-2-picoline (13d) and 5-Chloro-2-picoline (13e).

The synthesis of 3-iodo-2-PAM chloride (16m) and 5-iodo-2-PAM chloride (16n) was initiated with the iodonation of 2-picoline (19) to yield a mixture of 3-iodo-2-picoline (13m) and 5-iodo-2-picoline (13n). The isomers 13m and 13n could be separated chromatographically; however, because of the difficulty of separation, the mixture containing 13m and 13n was oxidized with I_2/DMSO to the corresponding aldehydes, 14m and 14n, respectively. The aldehydes 14m and 14n were separated chromatographically and converted to the oximes 15m and 15n. Alkylation of 15m and 15n with methyl iodide afforded the pyridinium oximes 16m and 16n, which were converted to the chloride salts 17m and 17n, using ion exchange chromatography (Dowex Cl^- form). Sufficient quantities of 3-iodo-2-PAM chloride (16m) and 5-iodo-2-PAM chloride (16n) were prepared and sent to WRAIR for in vivo testing in soman-challenged mice.

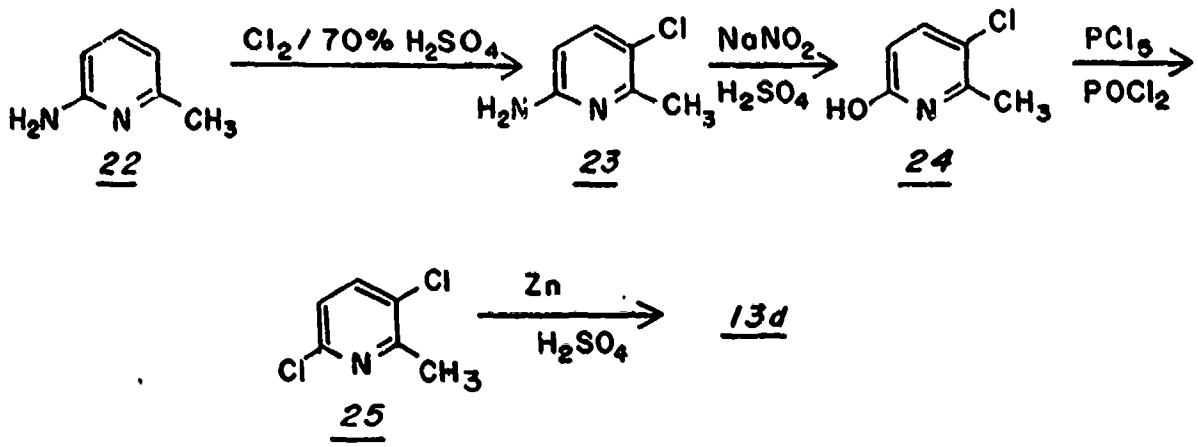


Figure 7. Synthesis of 3-Chloro-2-picoline (13d).

In our attempts to synthesize 3-cyano-2-PAM chloride (17f) and 5-cyano-2-PAM chloride (17g), we prepared the 3-cyano aldehyde 14f and the 5-cyano-aldehyde 14g from the corresponding 3- and 5-iodo aldehydes 14m and 14n (Figure 8). The 3- and 5-iodo aldehydes 14m and 14n were converted to the acetals 26 and 27, which were then treated with CuCN in dimethylformamide (DMF) to yield the 3- and 5-cyano aldehyde acetals 28 and 29, respectively. Deprotection of 28 and 29 with dilute HCl gave the 3- and 5-cyano aldehydes 14f and 14g, respectively. As an alternative synthetic procedure to 5-cyano aldehyde 14g, we converted 5-cyano-2-picoline (13g) and followed the synthetic steps outlined in Figure 5. The 5-cyano aldehyde 14g could be converted smoothly to 5-cyano-2-pyridine aldoxime (15g) in neutral aqueous hydroxylamine (Figure 8). Attempts to convert the 3-cyanoaldehyde 14f to the aldoxime 15f using identical conditions resulted

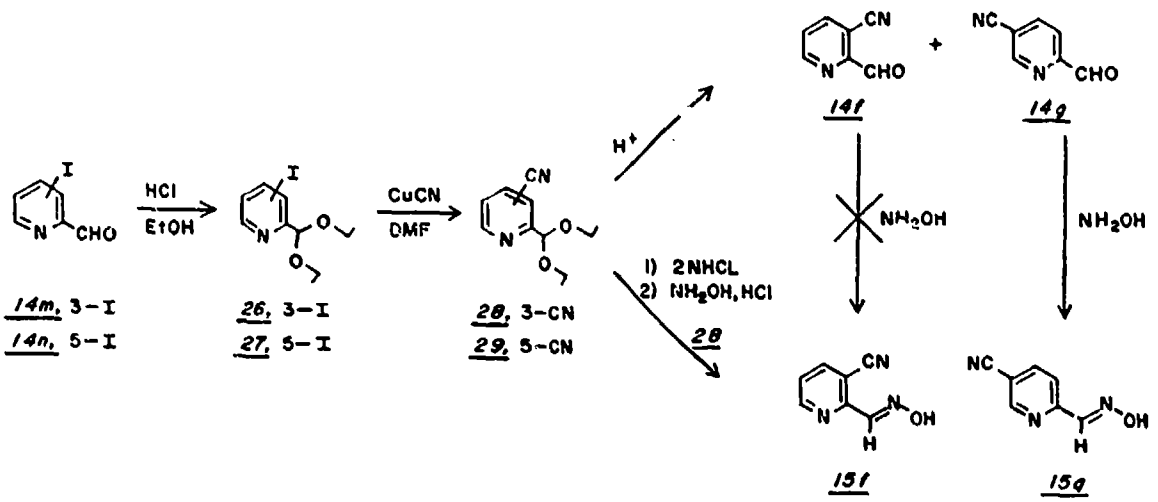


Figure 8. Syntheses of 3-Cyano (15f) and 5-Cyano (15g)-2-Pyridine Aldoximes.

in decomposition of the starting material (Figure 8). However, the aldoxime 15f could be prepared directly in a "one pot" reaction from the acetal 28 by reaction with aqueous HCl and NH_2OH. Attempts to methylate the 3-cyano aldoxime 15f with MeI, dimethylsulfate or methyl tosylate in the presence or absence of K_2CO_3 led to decomposition of the starting material. Attempts to methylate directly the 3-iodo acetal 26 and the 3-cyano acetal 28 also met with failure.

Methylation of the 5-cyano aldoxime 15g with methyl tosylate afforded the desired 5-cyano-2-PAM tosylate (16g), which was converted to the chloride salt 17g using ion exchange chromatography (Dowex Cl^-) (Figure 5). Sufficient quantities of 5-cyano-2-PAM chloride (17g) were prepared and sent to WRAIR for in vivo testing in soman-challenged mice.

The nitrile intermediates 28 and 29 did, however, provide us with potential routes to the synthesis of the 3- and 5-carboxamido oximes 17j and 17k. Controlled basic hydrolysis of 28 and 29 afforded the carboxamido acetals 30 and 31 (Figure 9). The 5-carboxamido acetal 31 was converted to the 5-carboxamido oxime 15k using the steps shown in Figure 9. As an alternative synthetic route to the 5-carboxamido oxime 15k, we oxidized methyl-6-methyl nicotinate (13i) with I_2/DMSO to the corresponding aldehyde 14i. The aldehyde 14i was converted to the corresponding oxime 15i by treatment with NH_2OH. Aminolysis of 15i afforded the desired 5-carboxamidopyridine aldehyde 15k (Figure 9). The oxime 15k was methylated with methyliodide to form the pyridinium aldoxime iodide 16k which was converted on ion exchange chromatography (Dowex Cl^-) to the chloride salt 17k (Figure 5). Sufficient quantities of 5-carboxamido-2-PAM chloride (17k) were prepared and sent to WRAIR for in vivo testing in soman-challenged mice.

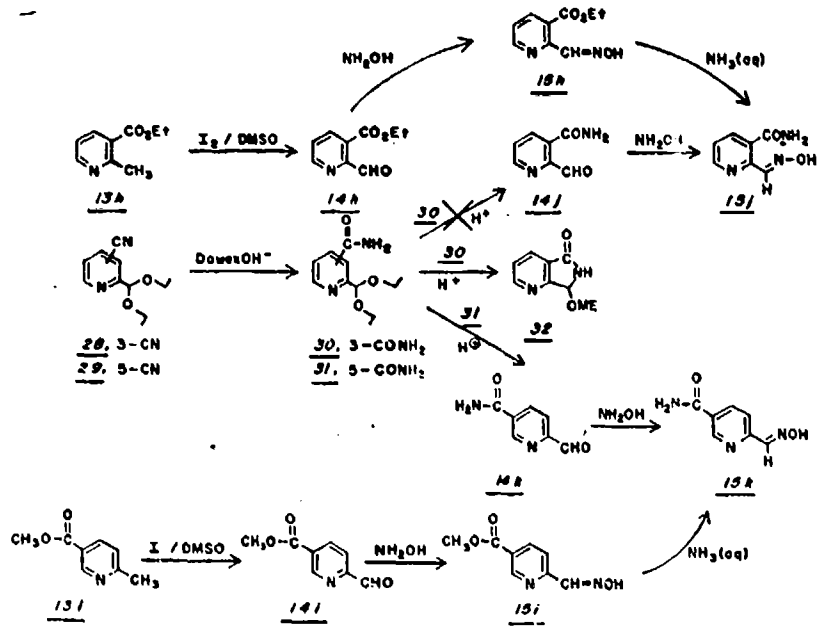
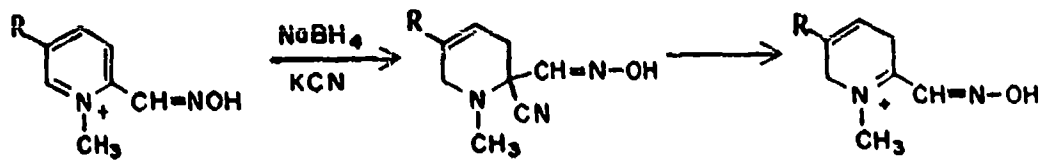


Figure 9. Syntheses of 3-Carboxamido (15j) and 5-Carboxamide (15k) Pyridine Aldoximes.

Attempts to prepare the corresponding 3-carboxamido pyridinium oxime 17j have been unsuccessful. Attempts to hydrolyze the acetal 30 to the aldehyde 14j resulted in the formation of 32 (Figure 9). As an alternative synthetic route to the 3-carboxamido aldehyde 14j, we oxidized ethyl-2-methylnicotinate (13h) with I_2/DMSO to the corresponding aldehyde 14h. The aldehyde 14h was converted to the corresponding oxime 15h upon treatment with NH_2OH. Aminolysis of 15h afforded the desired 3-carboxamido pyridine aldehyde 15j (Figure 9). Attempts to methylate the aldoxime 15j using methyliodide, dimethylsulfate and methyl tosylate failed.

Following the literature procedures outlined by Bodor et al.[21], we have prepared pro-2-PAM (8) from 2-PAM (3). This prodrug form of 2-PAM (3) was used in the biodistribution studies outlined in Section D,2 of this report. We have attempted to use the Bodor et al. procedure[21] as outlined in Figure 10 to prepare 5-iodo-pro-2-PAM (18n), 5-cyano-pro-2-PAM (18g) and 5-carboxamido-pro-2-PAM (18k). However, reduction of the corresponding 5-substituted pyridinium oximes 17n, 17g and 17k with $NaBH_4$ in the presence of KCN did not afford isolatable and identifiable products. Therefore, we were forced to abandon this prodrug approach for substituted pyridinium oximes.



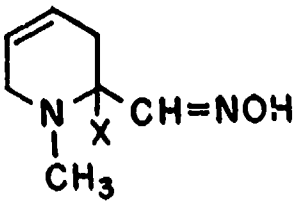
3 R=H
17g R=CN
17k R=$CONH_2$
17n R=I

33 R=H

8 R=H
18g R=CN
18k R=$CONH_2$
18n R=I

Figure 10. Attempted Synthesis of Dihydropyridinium and Tetrahydropyridinium Oximes as Prodrugs.

As another potential prodrug form of 2-PAM we isolated the cyanide addition product 33 (Figure 10). The yield of 33 from 2-PAM (3) was considerably less than that reported by Shek et al.[21] As a solid, prodrug 33 was not stable at room temperature. It was reasonably stable at room temperature when stored under nitrogen, but darkened considerably with time. Substitution of KSCN or KBr for KCN under identical reaction conditions (pH 1-2) did not yield the desired 34 and 35. The thiocyanate adduct 34 and bromide adduct 35 were isolated when the pH of the reaction mixture was raised to the 7-8 range. Yields of 40-60% were obtained and the desired adducts 34 and 35 were characterized spectrally.



34 X = SCN

35 X = Br

The tetrahydropyridinium prodrugs 33, 34 and 35 were monitored for conversion to 2-PAM (3) in phosphate buffer (150 mM, pH 6.5-8.5). The progress of the conversion was monitored with time via HPLC (data not shown). In this manner 2-PAM could be positively identified and quantitated by UV detection. The only compound which showed significant conversion to 2-PAM (3) was the cyanide adduct 33, but conversion was only on the order to 20%. The thiocyanate adduct 34 exhibited no measurable conversion to 2-PAM (3) whereas the bromide adduct 35 showed only a slight (5-10%) conversion to the parent compound 3.

2. Biology

Newly synthesized regenerators of AChE were evaluated in vitro using immobilized AChE[36] and the Ellman assay technique.[28] Eel AChE was attached via a stable Schiff base linkage to functionalized polyethylene beads using a modification of the procedure of Ngo, Laidler and Yam[40] as outlined in Figure 11.

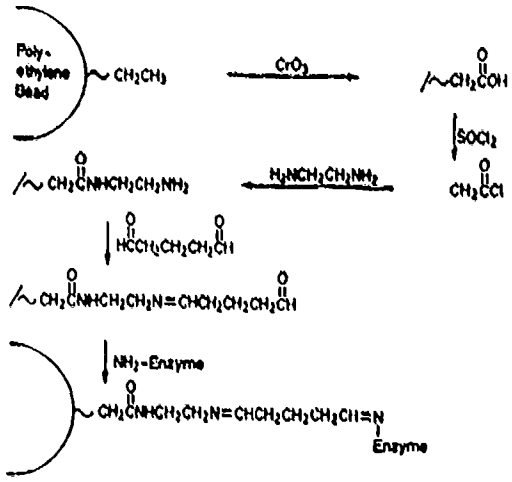


Figure 11. General Scheme for Immobilization of AChE on Polyethylene Beads.

Acetylthiocholine was used as a substrate and dithiobisnitrobenzoate (DTNB) as chromogen and the activity of AChE was followed spectrophotometrically at 412 nm.[36] A flow-through apparatus powered by a peristaltic pump was assembled (Figure 12) which allowed sequential and stepwise: (1) determination of initial immobilized enzyme activity; (2) inhibition with organophosphate; (3) reactivation with various oxime concentrations; (4) determination of regenerated enzyme activity; and (5) washing between steps to remove residuals.

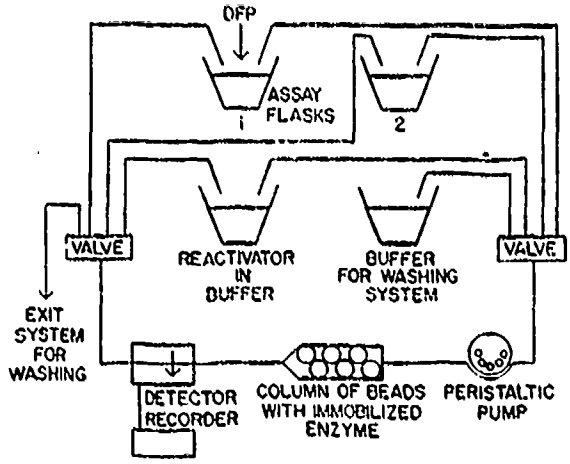
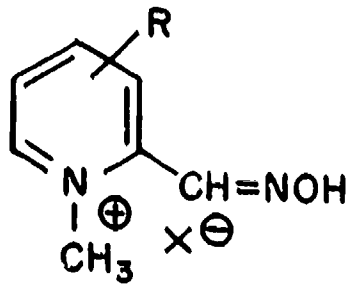


Figure 12. Schematic of Closed-Loop, Flow-Through System for Immobilized AChE Assay.

Flasks 1 and 2 contain substrate (ATC, 10^{-3}M) and chromogen (DTNB, 5 X 10^{-4}M) in 3-[N-morpholine]propanesulfonic acid (MOPS) buffer (0.1 M, pH 7.8) at 37°C. The peristaltic pump was run at a flow rate of 5.8 ml/minute and the detector was set at 412 nm.

Diisopropylfluorophosphate (DFP)-inhibited AChE could be regenerated using 2-PAM (3), TMB-4 (4) and MINA (6) as shown in Figures 13-15. The newly synthesized 3- or 5-substituted 2-PAM's were evaluated for their ability to regenerate DFP-inhibited AChE and the results are summarized in Table 1. It is interesting to note that all of the 3-substituted 2-PAM's (3-iodo, 3-bromo, 3-chloro, 3-methyl) were more potent as regenerators of DFP-inactivated AChE than 2-PAM. In contrast, the 5-substituted 2-PAM's were in general less effective in regenerating the inactivated enzyme.

Table 1. Effects of 3- or 5-Substituted 2-PAM's as Regenerators of DFP-Inactivated AChE.



Compound	R=	Reactivation[a]	% Reactivation Relative to 2-PAM
2-PAM	H	53	100
3-iodo-2-PAM	3-I	64	121
5-iodo-2-PAM	5-I	25	47
3-bromo-2-PAM	3-Br	77	145
5-bromo-2-PAM	5-Br	37	70
3-chloro-2-PAM	3-Cl	88	166
5-chloro-2-PAM	5-Cl	49	92
3-methyl-2-PAM	3-CH_3	69	130
5-methyl-2-PAM	5-CH_3	57	107
5-cyano-2-PAM	5-CN	28	53
5-carboxamido-2-PAM	5-$CONH_2$	36	70

[a]Immobilized eel AChE was inhibited with DFP (6×10^{-4} M), then washed free of excess organophosphate.[16] The DFP-inactivated AChE was incubated with 10^{-3} M oxime at 37° for 5 minutes. After exposure to the oxime, the enzyme was washed free of regenerator, and AChE activity measured.

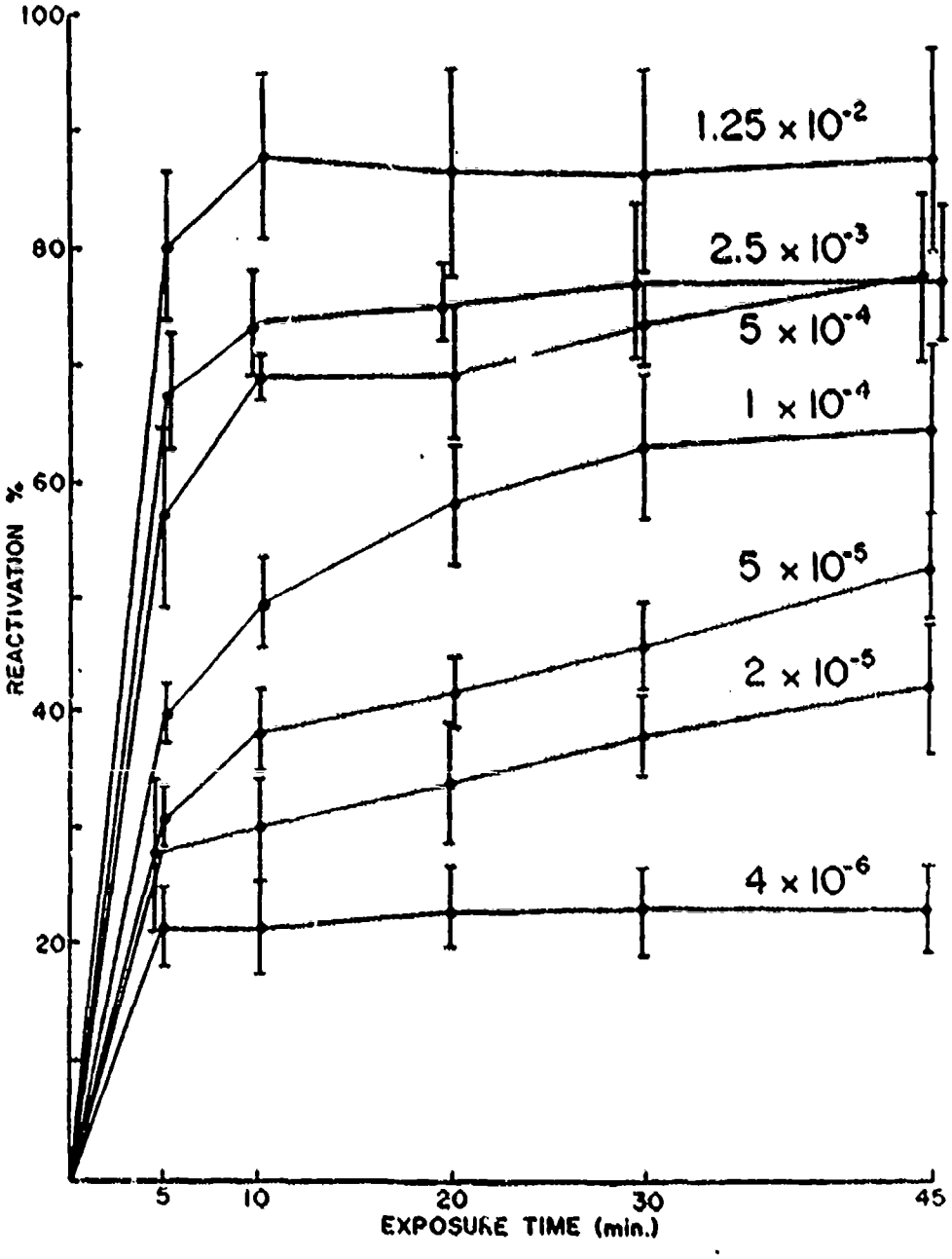


Figure 13. Effects of 2-PAM on Regeneration of Immobilized Eel AChE.

Concentrations of oxime expressed in molarity. The Enzyme was inhibited with DFP (6 X 10^{-4}M), then washed free of excess DFP. Enzyme was then exposed to varying concentrations of 2-PAM for time points shown. After each exposure to 2-PAM, the enzyme was washed free of regenerator, esterase activity measured, and exposure to regenerator resumed. These steps were repeated until the enzyme had been exposed to regenerator for a total of 45 minutes.

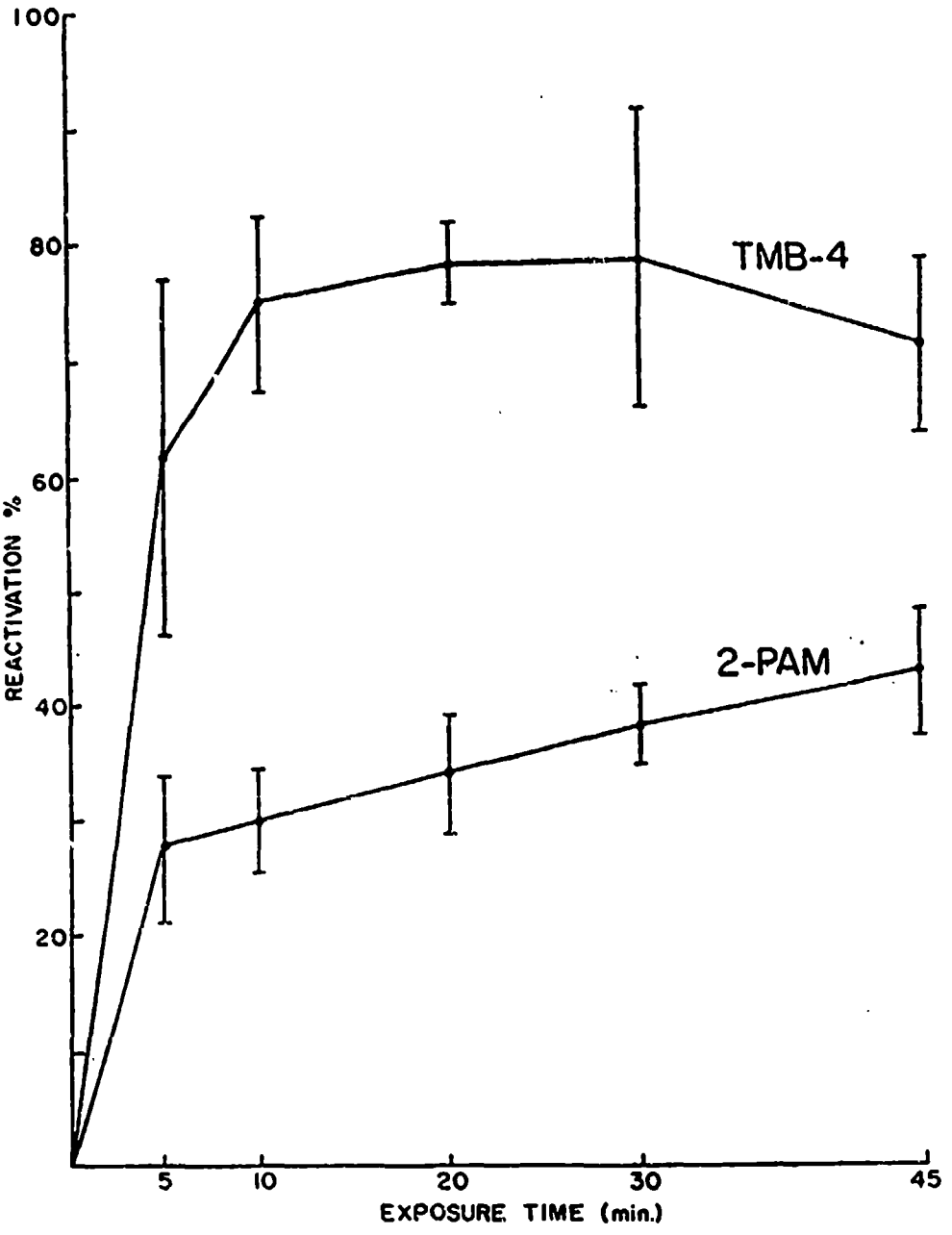


Figure 14. 2-PAM vs. TMB-4 as Regenerator of DFP-Inhibited Immobilized Eel AChE.

Concentrations were 2 X 10^{-5}M. Conditions as described in Figure 12.

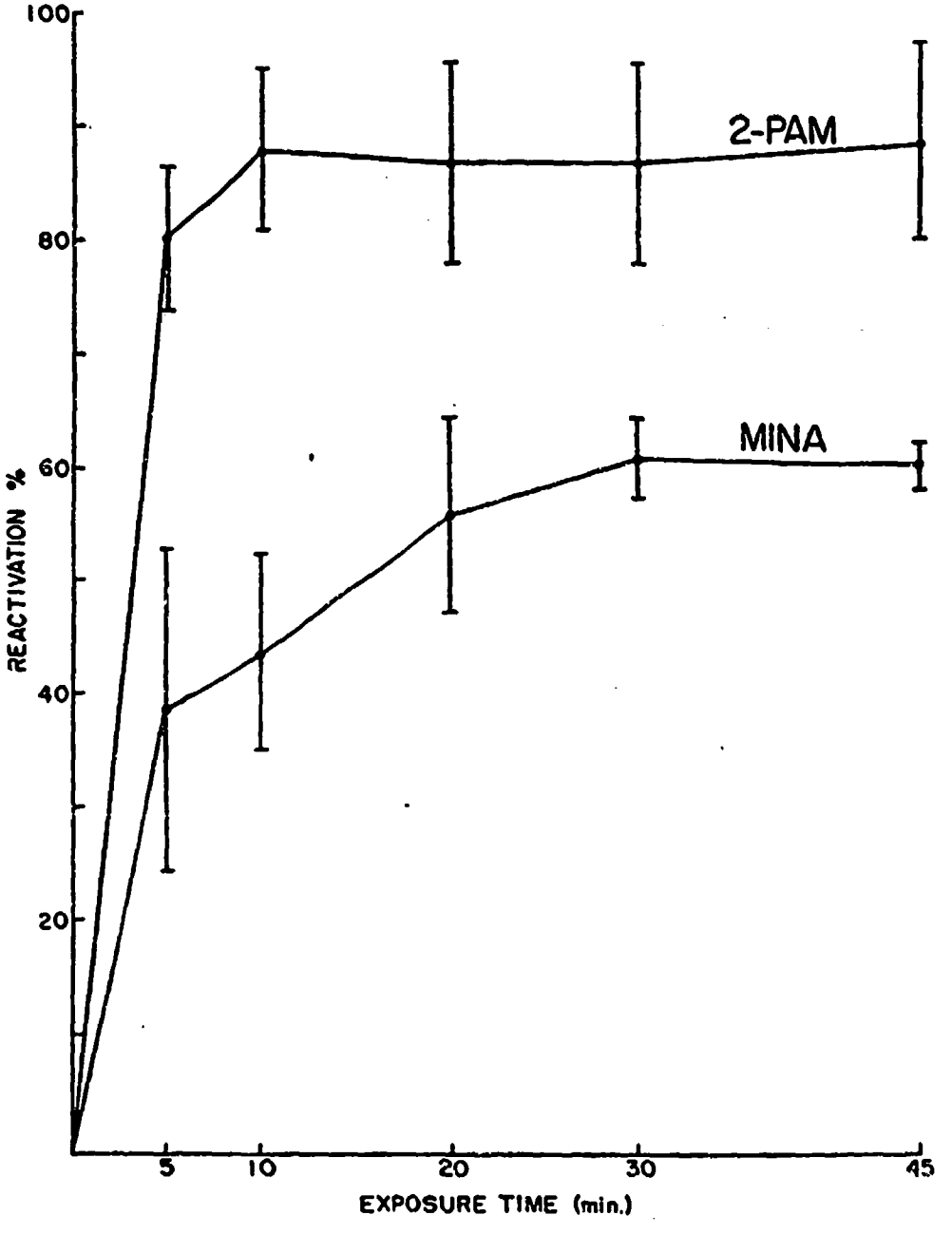
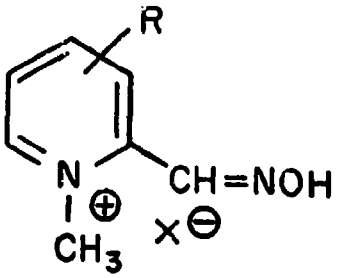


Figure 15. 2-PAM vs. MINA as Regenerator of DFP-Inhibited Immobilized Eel AChE.

Concentrations were 1.25 X 10^{-2}M. Conditions as described in Figure 12.

Experiments were also carried out to determine the ability of the 3- or 5-substituted 2-PAM's to save mice challenged with DFP. Following a dose (s.c.) of 2 X LD_{50} of DFP, mice were immediately administered (i.m.) various doses of the 3- or 5-substituted 2-PAM's. The results shown in Table 2 indicate that the most potent analog in vivo against a DFP challenge is the 5-iodo-2-PAM. However, 3-iodo-2-PAM, 3-bromo-2-PAM and 5-bromo-2-PAM also showed significant activity. The potent activity of the 5-iodo-2-PAM and 5-bromo-2-PAM have been confirmed through independent studies in soman-challenged mice conducted by WRAIR personnel.

Table 2. ED_{50}[a] Values for 3- or 5-Substituted Pyridinium Oximes and 2-PAM in DFP-Challenged Mice.[b]

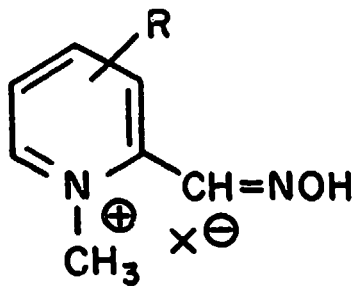


Compound	R=	ED_{50} µmol/kg (95% C.L.)	ED_{50} mg/kg (95% C.L.)
2-PAM	H	46 (62-35)	7.9 (11-6)
3-iodo-2-PAM	3-I	30 (40-22)	8.8 (12-6.7)
5-iodo-2-PAM	5-I	1.2 (2.3-0.6)	0.35 (0.69-0.18)
3-bromo-2-PAM	3-Br	59 (77.5-44)	14.9 (19.5-11)
5-bromo-2-PAM	5-Br	58 (88-39)	14.7 (22-9.8)
3-chloro-2-PAM	3-Cl	211 (237-188)	44 (49-39)
5-chloro-2-PAM	5-Cl	225 (267-191)	46.5 (55-39.5)
3-methyl-2-PAM	3-CH_3	252 (325-196)	47 (61-36.5)
5-methyl-2-PAM	5-CH_3	187 (224-156)	35 (42-29)
5-cyano-2-PAM	5-CN	--	
5-carboxamido-2-PAM	5-CONH	105 (122-88)	22.6 (26.3-19.0)
pro-2-PAM[c]		117 (186-73)	20.3 (32-13)

[a]Oxime dose required to obtain 50% survival at 24 hr.
[b](2 X LD_{50}, s.c., water), oxime (i.m., water, 1 ml/kg inj. vol.).
[c]i.v., 50 mM citrate, pH 3.5.

In an attempt to correlate the in vitro and in vivo biological effects of the 3- or 5-substituted 2-PAM's with some physico-chemical parameter, we have determined the partition coefficients (octanol/water) and the pKa values for the oxime functionality. The results of these experiments are shown in Table 3. It should be noted that 5-iodo-2-PAM and 5-bromo-2-PAM, which were the most potent analogs in soman-challenged mice, also have octanol/water partition coefficients which would favor CNS permeability and the pKa values for the oxime functional group are in an acceptable range for optimal reactivation of organophosphate-inactivated AChE.

Table 3. pKa and Partition Coefficients for 3- and 5-Substituted 2-PAM's.



Compound	R=	pKa[a]	Octanol/H_2O Partition Coeff.[a]
2-PAM	H	7.9	0.0012
3-iodo-2-PAM	3-I	7.8	0.01
5-iodo-2-PAM	5-I	7.5	0.01
3-bromo-2-PAM	3-Br	7.8	0.004
5-bromo-2-PAM	5-Br	7.5	0.01
3-chloro-2-PAM	3-Cl	7.9	0.005
5-chloro-2-PAM	5-Cl	7.6	0.004
3-methyl-2-PAM	3-CH_3	8.3	0.001
5-methyl-2-PAM	5-CH_3	8.2	0.0004
5-cyano-2-PAM	5-CN	6.8	0.002
5-carboxamido-2-PAM	5-$CONH_2$	7.3	0.0002
pro-2-PAM		6.3, 10.5	0.45

[a]See Experimental Methods, Section G, for experimental details.

In addition to using the immobilized AChE to evaluate regenerators of AChE, the immobilized AChE also has the potential to be used for studying the effects of organophosphates. For example, we have studied the effects of organophosphates (DFP, paraoxon, soman) on the activity of immobilized eel AChE and human RBC AChE and the results are summarized in Figure 16. With the eel enzyme, the order of sensitivity to organophosphates was soman >> paraoxon > DFP. The immobilized RBC AChE exhibited distinct differences from the immobilized eel AChE in response to organophosphates. As shown in Figure 16, the RBC AChE was less sensitive than the eel AChE to soman at concentrations greater than 5×10^{-9} M. The RBC AChE was slightly more sensitive to DFP than eel AChE at all concentrations tested. Against DFP inactivation, RBC AChE was less reactivated (26%) than eel AChE (53%) by 2-PAM (10^{-3} M) during a 5 minute incubation. We also observed that the DFP-inactivated RBC and eel enzymes underwent aging at approximately the same rates (0.57% activity loss and 0.41% activity loss, respectively).

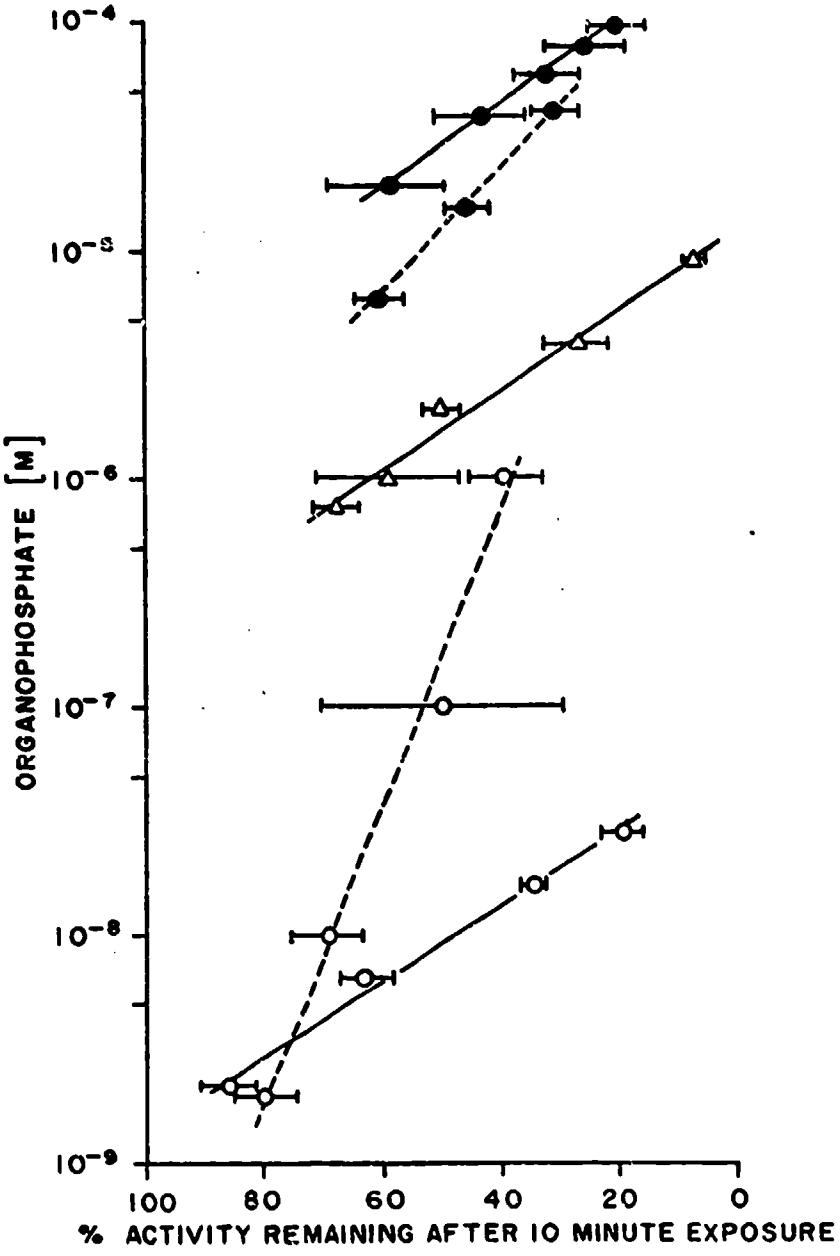


Figure 16. Inactivation of Immobilized RBC and Eel AChE to Organophosphates. Eel AChE (———), RBC (- - -), DFP (●), Paraxon (△) and Soman (○). See Experimental Methods for experimental details.

Our laboratory has also developed a new and efficient HPLC assay for regenerators of AChE. This assay has been used to monitor the tissue levels of both 2-PAM and its prodrug form, pro-2-PAM.

The HPLC systems developed in this study utilize a commercially available, polymer-backed, reversed-phase column that allows the use of both strongly acidic and strongly basic buffers (pH range 1 to 13). Figures 17 and 18 show that these HPLC systems produce separations that allow for detection and quantification of 2-PAM or pro-2-PAM in biological tissues.

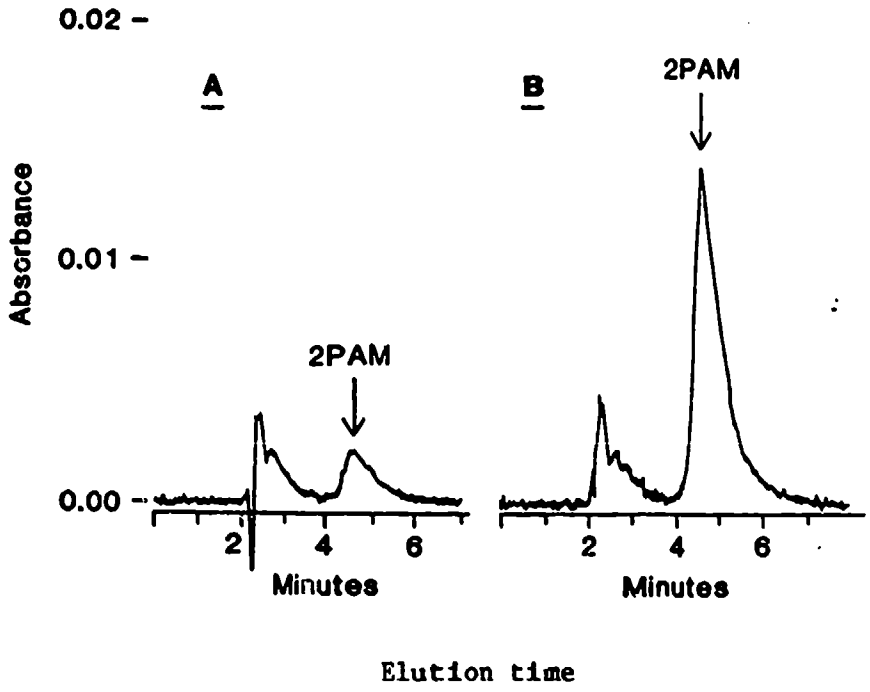


Figure 17. HPLC Elution Pattern of 2-PAM.

Typical elution profiles of 2-PAM from a dose of 2-PAM (A) (i.m., 50 mg/kg) and 2-PAM from a dose of pro-2-PAM (B) (i.v., 50 mg/kg) in brain tissue samples using a basic solvent system (100% Na_2CO_3 buffer, 0.1 M, pH = 10.5) and chromatography on a PRP-1 reversed-phase column. 20 µl injection; flow rate = 1.0 ml/min; detector wavelength = 334 nm.

These HPLC systems were used to determine the CNS permeability of pro-2-PAM and the extent of its conversion to 2-PAM in the brain. Animals were given injections of either 2-PAM (i.m., 50 mg/kg) or pro-2-PAM (i.v., 50 mg/kg) and then sacrificed at various times up to 15 minutes after injection and the brain levels of 2-PAM or pro-2-PAM determined, using the HPLC systems illustrated in Figures 17 and 18. Four important observations can be made about the data shown in Figure 19; (a) Two minutes after administration of pro-2-PAM, the ratio of pro-2-PAM to 2-PAM in the brain

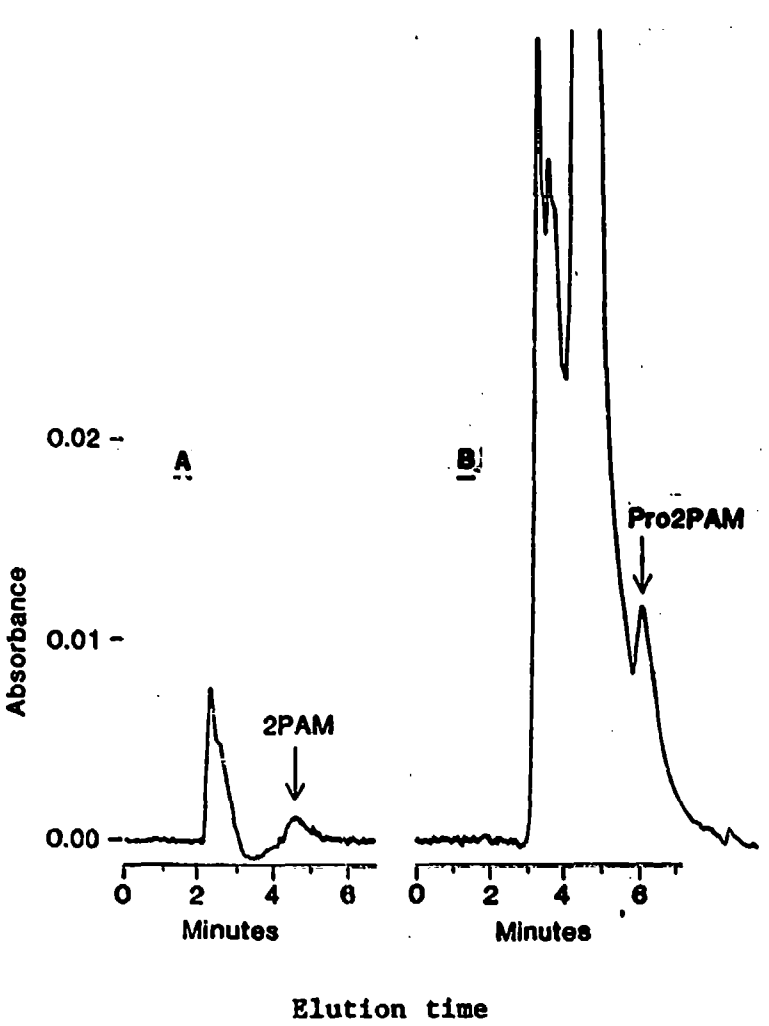


Figure 18. HPLC Elution Pattern of 2-PAM and Pro-2-PAM.

Typical elution profiles of 2-PAM (A) and pro-2-PAM (B) from a dose of pro-2-PAM (i.v., 50 mg/kg) in brain tissue samples, using an acidic solvent system (100% H_3PO_4 buffer, 0.1 M, pH = 2.5) and chromatography on a PRP-1 reversed phase column. 20 µl injection; flow rate 1.0 ml/min; detector wavelength = 312 nm (A), 251 nm (B).

is approximately 2:7 (2.04 prodrug vs. 0.76 2-PAM % dose/gm brain); (b) 15 minutes after administration of the prodrug, there are still significant levels of pro-2-PAM (2.12% dose/gm brain); (c) administration of pro-2-PAM produces a higher brain level of 2-PAM (5 times) than that achieved with a comparable dose of 2-PAM; however, the magnitude of the increase is less than the 13-fold increase reported earlier by Shek *et al.*[23]; and (d) the combined brain levels of pro-2-PAM and 2-PAM from a dose of the prodrug is 20 times higher at 2 minutes after drug administration than the brain level of 2-PAM from a dose of 2-PAM (2.8 vs. 0.14%, dose/gm brain).

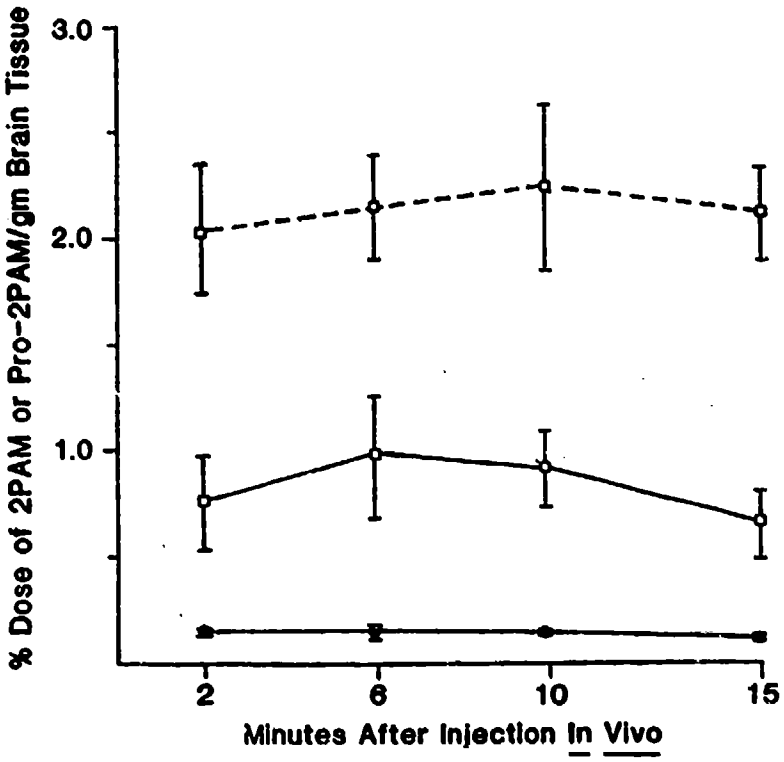


Figure 19. Brain Levels of 2-PAM and of Pro-2-PAM.

Animals were given a 50 mg/kg dose of 2-PAM (i.m.) or pro-2-PAM (i.v.) and brain levels of 2-PAM and pro-2-PAM measured. 2-PAM injection: 2-PAM samples were analyzed in the basic solvent system (●—●, 0.1 M Na_2CO_3, pH = 10.5, 334 nm). Pro-2-PAM injection: Brain was quick-frozen and dissected in an acid bath to prevent conversion of pro-2-PAM to 2-PAM during analysis. Samples were analyzed in the acidic solvent system (0.1 M H_3PO_4, pH = 2.5) for pro-2-PAM remaining (□---□, 250 nm) and 2-PAM formed from pro-2-PAM (□—□, 312 nm).

Analysis of blood taken from the sacrified animals showed that a dose of pro-2-PAM delivers 2-PAM mainly to RBC's, whereas a dose of 2-PAM is distributed mainly in the plasma (Figure 20). These results are consistent with those reported earlier by Shek et al.[23]

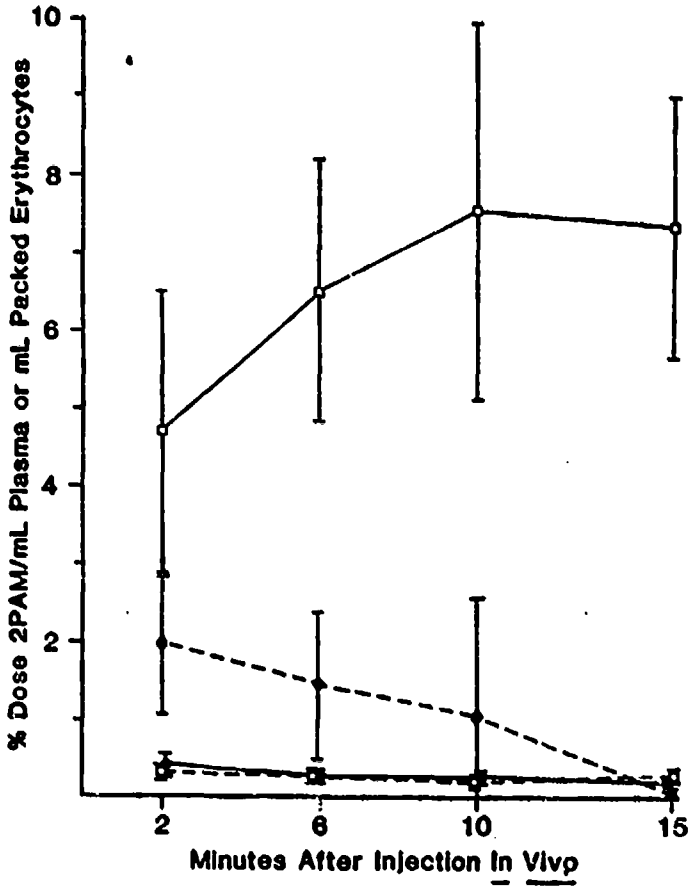


Figure 20. RBC and Plasma Levels of 2-PAM and of Pro-2-PAM.

Animals were given a 50 mg/kg dose of 2-PAM (i.m.) or pro-2-PAM (i.v.) and RBC and plasma levels of 2-PAM and pro-2-PAM measured. 2-PAM injection: RBC (●—●) and plasma (●- -●). Pro-2-PAM injection: RBC (□—□) and plasma (□- -□). All samples were analyzed in basic solvent system (0.1 M Na_2CO_3, pH = 10.5, 334 nm).

Analysis of the kidneys from sacrificed animals showed that at 2 minutes the kidney level of 2-PAM from a dose of 2-PAM was significantly higher than the level of 2-PAM from a dose of pro-2-PAM (Figure 21). By 6 minutes the kidney levels of 2-PAM from the two dosage forms were essentially equivalent.

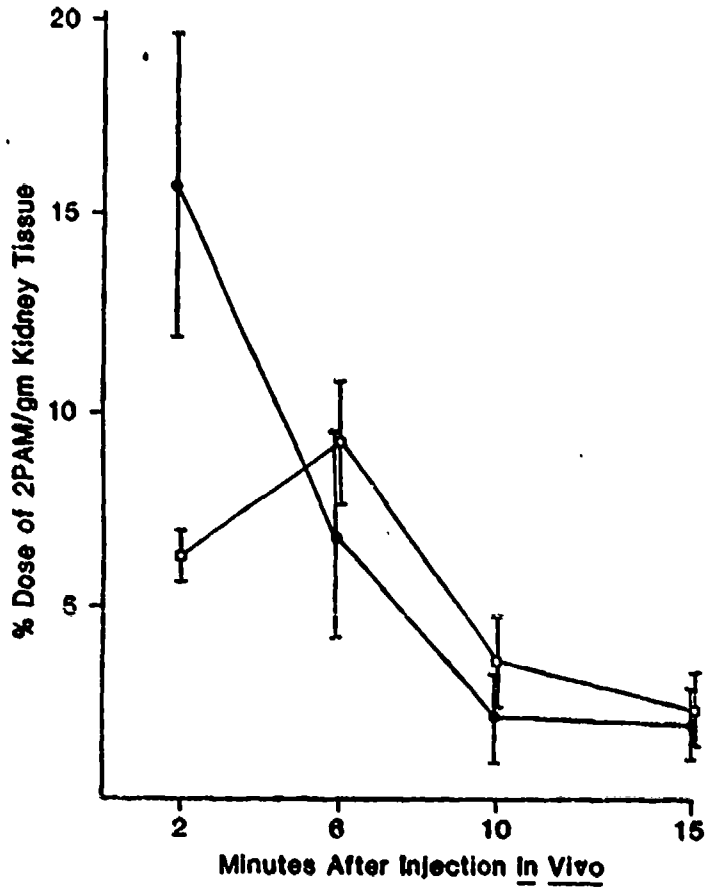


Figure 21. Kidney Levels of 2-PAM and of Pro-2-PAM.

Animals were given a 50 mg/kg dose of 2-PAM (i.m.) and pro-2-PAM (i.v.) and kidney levels of 2-PAM and pro-2-PAM measured. 2-PAM injection (●—●) and pro-2-PAM injection (□—□) were both analyzed as 2-PAM in basic solvent (0.1 M Na_2CO_3, pH = 334 nm).

In order to explain the apparent stability of pro-2-PAM in brain tissue (Figure 19), we studied the oxidation of pro-2-PAM to 2-PAM in physiological buffers. As shown in Table 4, the rate of air oxidation of the prodrug to 2-PAM is inversely proportional to the initial concentration of pro-2-PAM. As shown in Table 5, the maximum percent conversion of the prodrug to 2-PAM in the presence of $AgNO_3$ is also inversely proportional to the initial concentration of pro-2-PAM.

Table 4. Rate of Pro-2-PAM Conversion to 2-PAM in a Physiological Buffer[a].

pro-2-PAM (M)	$t_{1/2}$ (min)
10^{-5}	3.2
10^{-4}	10.0
10^{-3}	16.5
10^{-2}	31.3

[a]Experiments were carried out in MOPS buffer (0.1 M, pH = 7.4) containing bovine serum (1/1). At various times perchloric acid was added to samples. The samples were immediately filtered and analyzed by HPLC, using the acidic buffer system (0.1 M H_3PO_4, pH = 2.5). 2-PAM was detected at 312 nm.

Table 5. Extent of pro-2-PAM Conversion to 2-PAM In Vitro[a].

pro-2-PAM (M)	Maximum Conversion (%)
10^{-5}	86
10^{-4}	70
10^{-3}	41
5×10^{-2}	31

[a]pro-2-PAM samples were oxidized with excess $AgNO_3$ at pH > 7. After 20 minutes the samples were filtered and analyzed by HPLC, using the basic buffer system (0.1 M Na_2CO_3, pH 10.5). 2-PAM was detected at 334 nm.

Based on the data shown in Tables 4 and 5, we predict that the percentage of dose per gram of brain tissue should be inversely proportional to the dose of pro-2-PAM administered. This hypothesis was confirmed by the data shown in Table 6. Although the brain levels of 2-PAM increased with increasing doses of pro-2-PAM, the percentage of dose in the brain is inversely proportional to the dose of the prodrug.

Table 6. Dependence of the 2-PAM Brain Levels on the Dose of Pro-2-PAM.

Pro-2-PAM Dose mg/kg	2-PAM Present in Brain[a]			
	2 Minutes		6 Minutes	
	µg/gm tissue	% dose/gm tissue	µg/gm tissue	% dose/gm tissue
100	32.6 (44.1-21.0)[2]	0.94 (1.33-0.55)	46.9 (56.1-37.6)	1.33 (1.55-1.10)
50	26.1 (36.1-16.2)	1.54 (2.03-1.04)	28.8 (34.1-23.6)	1.75 (2.09-1.41)
10	7.1 (8.4-5.8)	1.90 (2.22-1.59)	6.8 (7.7-5.9)	1.91 (2.09-1.73)

[a]Groups (n=3) of mice were given either 10, 50 or 100 mg/kg doses of pro-2-PAM (i.v.). The animals were sacrificed at 2 minutes or 6 minutes after drug administration. The brains were quickly frozen in a -70° C dry ice bath and dissected under acidic conditions to prevent conversion of the prodrug to 2-PAM. Samples were then analyzed by HPLC, using the acidic solvent system (0.1 M H_3PO_4, pH = 2.5). 2-PAM was detected at 312 nm.

[b]Confidence limits.

Our data, as well as literature data (Table 7), suggest that 2-PAM is more effective than pro-2-PAM in protecting animals from an organo-phosphate challenge. These results are inconsistent with the observation that the prodrug produces a higher brain level of 2-PAM. As shown in Figure 19, the prodrug also produces elevated brain levels of pro-2-PAM, but this dihydropyridine oxime probably does not contribute to the reactivation of brain AChE. It is noteworthy that pro-2-PAM given prophylactically 10 minutes prior to organophosphate exposure (Table 7) shows a better protective effect than 2-PAM. These results might suggest that the $t_{1/2}$ for conversion of the prodrug to 2-PAM in biological tissues is longer than was previously anticipated, a result which is consistent with the data shown in Table 4.

Table 7. Literature Reports of the Protective Ability of Pro-2-PAM and 2-PAM against Organophosphate Challenge in Mice.

	2-PAM	pro-2-PAM	Ratio	Ref.
ED_{50}[a] (mg/kg)	7.9	20.3	2.5	b
95% confidence limit	(6.0-10.6)	(12.7-32.4)		
ED_{50} (mg/kg)	3.8	11.6	3.0	41[c]
S.E.	±0.8	± 3.0		
#Died/#Tested	0/6	3/6	--	42[d]
Protective Ratio[e] (Oxime Administered Prophylactically)	1.41	2.20	0.64	29[f]

[a]Dose required to obtain 50% survival at 24 hr.
[b]Performed in this lab: 2-PAM i.m., pro-2-PAM i.v.; vs. DFP (6.0 g/kg, s.c.).
[c]i.p. injection of oximes; vs. paraoxon (0.9 mg/kg, s.c.).
[d]34 mg/kg i.p. oxime dose; vs. paraoxon (8 μmole s.c.).
[e]Protective Ratio: LD_{50} of prophylaxis and organophosphate/LD_{50} of organophosphate.
[f]50 mg/kg, i.m,. oxime dose 10 minutes before DFP (s.c.) challenge.

E. Conclusions

1. Chemistry

a) Various 5-substituted 2-PAM's (I, Br, Cl, CH_3, CN, $CONH_2$-substituted) have been synthesized and characterized.

b) Various 3-substituted 2-PAM's (I, Br, Cl, CH_3-substituted) have been synthesized and characterized.

c) Attempts to prepare the 3-cyano and 3-carboxamido 2-PAM's have been unsuccessful because of the inherent reactivity of these moieties and the possibility for intramolecular reactions with the neighboring oxime.

d) The 3-iodo, 3-bromo and 3-chloro-2-PAM's were shown to be unstable in aqueous solution, apparently because of an intramolecular cyclization reaction involving the adjacent oxime.

e) With the exception of 5-cyano-2-PAM (pKa = 6.8), the 3- and 5-substituted 2-PAM's synthesized in this study had pKa's for the oxime moieties in the optimal range (7.3-8.3) for reactivation of organophosphate-inactivated AChE.

f) With the exception of 3-methyl and 5-methyl-2-PAM's, the 3- and 5-substituted 2-PAM's synthesized in this study had improved octanol/H_2O partition coefficients.

g) Pro-2-PAM has been synthesized from 2-PAM using the procedures reported by Bodor et al.[21] However, attempts to prepare various 5-substituted pro-2-PAM's (I, CN, $CONH_2$ substituted) have failed apparently because of the inherent chemical instability of the substituted dihydropyridinium oximes.

h) In vitro studies showed that the rate and extent of pro-2-PAM oxidation to 2-PAM was dependent on the reaction conditions and the initial concentration of the dihydropyridinium species.

i) Tetrahydropyridinium oximes have been synthesized as potential prodrugs of 2-PAM, but only the cyanide addition product showed any potential for regenerating the parent oxime.

2. Biology

a) When tested in vitro as regenerators of DFP-inactivated AChE, several of the 3- or 5-substituted 2-PAM's were more potent than 2-PAM. Order of potency: 3-chloro-2-PAM > 3-bromo-2-PAM > 3-methyl-2-PAM $\geq$ 3-iodo-2-PAM > 5-methyl-2-PAM $\geq$ 2-PAM $\geq$ 5-chloro-2-PAM > 5-carboxamido-2-PAM $\geq$ 5-bromo-2-PAM > 5-cyano-2-PAM $\geq$ 5-iodo-2-PAM.

b) When tested *in vivo* for their ability to protect mice from a challenge dose ($2 \times LD_{50}$) of DFP, several of the 3- or 5-substituted 2-PAM's were more potent than 2-PAM. Order of potency: 5-iodo-2-PAM >> 3-iodo-2-PAM $\geq$ 2-PAM > 3-bromo-2-PAM = 5-bromo-2-PAM > 5-carboxamido-2-PAM > 5-methyl-2-PAM > 3-methyl-2-PAM = 3-chloro-2-PAM = 5-chloro-2-PAM >> 5-cyano-2-PAM.

c) When tested *in vivo* by WRAIR for their ability to protect mice from a challenge dose ($2 \times LD_{50}$) of soman, 5-iodo-2-PAM and 5-bromo-2-PAM were more potent than 2-PAM.

d) There does not appear to be a good correlation between a compound's ability to regenerate DFP-inactivated AChE *in vitro* and its ability to protect mice from exposures to DFP or soman *in vivo*. This may be due to inherent differences in the oxime's ability to regenerate eel AChE versus mouse AChE, or it may be due to differences in distribution and metabolism of the oximes *in vivo*.

e) Using a newly developed HPLC assay, 2-PAM and pro-2-PAM can be detected in brain and other biological tissues (e.g., kidney) and biological fluids (e.g., blood).

f) Pro-2-PAM can be detected in brain after administration of pro-2-PAM. Pro-2-PAM delivers more 2-PAM to the brain than a dose of the parent drug (2-PAM), but less than previously reported by Bodor *et al.*[21] The differences are due to improved sample handling in our laboratory, by which we prevent the air oxidation of pro-2-PAM in biological samples during the workup procedure. This new mthodology has led to a more accurate estimate of the brain levels of 2-PAM and pro-2-PAM.

g) The brain level of 2-PAM is proportional to the dose of pro-2-PAM administered. However, the higher the dose of the prodrug, the lower the percentage of the dose delivered to the brain.

F. Recommendations

1. Chemistry

a) Based on the results of our studies, structural modifications of 2-PAM could lead to more efficacious regenerators of AChE. Additional 3- or 5-substituted 2-PAM's should be synthesized in order to establish a predictable structure-activity relationship for this series of compounds.

b) The dihydropyridinium oxime approach to prodrugs of 2-PAM appears to be of limited therapeutic utility. Therefore, further synthetic efforts in this area do not appear warranted.

2. Biology

a) Based on the results obtained to date with 5-iodo-2-PAM and 5-bromo-2-PAM, more extensive pharmacological and toxicological studies should be conducted with these analogs so as to evaluate their therapeutic potential.

b) Other immobilized forms of AChE (e.g., human RBC, mouse brain) should be evaluated as in vitro screening assays for potential AChE regenerators. Additional effort is needed in this area in order to arrive at a more accurate in vitro predictor of the potential efficacy of a regenerator.

c) Additional effort should be directed toward studying the biodistribution in vivo of pyridinium oximes.

d) Additional effort should be directed toward studying how pyridinium oximes permeate biological membranes (e.g., blood brain barrier).

e) Results of the studies outlined above should lead to the rational design of regenerators of AChE.

G. Experimental Methods

1. Equipment and Reagents

Electron impact mass spectra were recorded on either a Varian-MAT CH-5 or a Riber R-10-10 mass spectrometer with RDS data system for computer analysis and spectra printout. NMR spectra were obtained with either a Varian T-60, Hitachi Perkin-Elmer R-24B or Varian FT-80a and were run in 1% TMS/$CDCl_3$ unless otherwise noted. The IR spectra were obtained on either a Beckman IR-33 or an AccuLab-4 spectrometer and samples were run as either neat films or KBr pellets (1:100). UV-vis spectra were recorded on either a Cary 219 or a Beckman DU-5 spectrophotometer. HPLC determinations were performed on a Beckman 342 system (112 pumps, 420 controller and 340 organizer), a Kratos 769Z variable UV detector and a Spectra-Physics 4270 recording integrator. Separations were performed on the following reversed-phase columns: a 5 mm x 15 cm Ultrasphere 5 μm ODS RP, a 5 mm x 10 cm BrownLee Spheri-5 5 μm RP, and a 5 mm x 20 cm BrownLee PRP-1 10 μm RP, all with matching 3 cm guard columns. Normal phase analysis was performed on an Alltech 0.5 x 25 cm silica gel 10 μm column with 3 cm guard column and preparative scale purifications on an Alltech 1 x 25 cm RSIL 10 μm column with a 5 cm guard column. Ionization constants and pH adjustments were performed potentiometrically using an Orion Research model 399A pH meter equipped with a gel-filled combination electrode. Melting points were obtained as either capillary melting points (uncorrected) on a Thomas-Hoover apparatus or as micromelting points (corrected) on a Fisher-Johns melting point stage. The AChE assay was equipped with a Gilson Minipuls 2 variable speed peristaltic pump and a Gilson HM Holochrome variable UV-Vis flow detector.

The chemicals and solvents were generally reagent or HPLC (chromatography) grade unless purity was not crucial. Thin layer chromatography TLC) was carried out on Analtech SG GHLF 250 μm, Analtech Woelm SGF 1000 μm or EM SG-60 F254 200 μm plates. Liquid chromatography (LC) sorbents were either Brinkman EM SG-60 70-230 mesh or Woelm silica 63-200 mesh. Acetylcholinesterase (electric eel organ) was obtained commercially purified from either Worthington (1000-1400 units/mg) or Boehringer Mannheim (1000 units/mg). Acetylthiocholine, DTNB, MOPS, DFP, diethyl-p-nitrophenylphosphate (paraoxon) and TMB-4 (4) were obtained from Sigma and were used without further purification. DFP degrades slowly, even when stored at 4°C, and was replenished with a fresh supply at regular intervals to insure consistency. The low density polyethylene beads (4mm, precision) were supplied by Precision Plastic Ball Co., Chicago, IL. MINA was purchased from Pfaltz & Bauer, but required purification to homo-geneity by LC (SG Woelm, 8% acetone/methylene chloride v/v). Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and are within 0.4% of theoretical values unless otherwise noted.

a. Ionization Constant Determinations

The general procedure required the accurate preparation of .005-.01 M solutions of pyridinium oximes. These oxime solutions were then acidified to pH 3 with 0.005 M hydrochloric acid and titrated with 0.005 M sodium hydroxide. The pH was monitored potentiometrically with a pH meter and combination electrode. Data plots of pH vs. volume of sodium hydroxide

added were constructed and pKa's read from titration inflection points.

Partition Coefficient Determinations

The partition coefficients were determined by taking a 1.5 ml solution of quaternary oxime (10^{-5} - 10^{-3} M) in either deionized water or 0.1 M MOPS (pH 7.4) and vortexing for 0.5 minute with an equal volume of octanol (spectrograde, presaturated with water). The phases were separated and clarified by centrifugation and the concentrations in octanol determined spectrophotometrically.

2. Chemistry

3-Bromo-2-picoline (13c) and 5-Bromo-2-picoline (13l)

The method of van der Does[37] was employed to prepare 3-bromo-2-picoline (13c) and 5-bromo-2-picoline (13l). Four 250 ml pressure vessels were each filled with 100 ml of 65% oleum, 22 ml (220 mmol) of 2-picoline and 10.5 ml (204 mmol) of Br_2. After 16 hours of reaction, followed by sodium carbonate neutralization and vacuum distillation, the yield was 88 g (63%) of a light yellow-green oil, b.p. 45-60° C, 3 mm. Literature b.p. 71-73°C, 14 mm.[37] Analytical samples of each isomer 13c and 13l were prepared by column chromatography (silica gel, load ratio 1:200, eluted with 10% ethyl acetate/methylene chloride v/v), R_f 0.25 (13c) and 0.30 (13l).
13c - oil; IR (film): 3040 cm^{-1} (Ar-H), 2980 (CH_2-H); NMR: δ 2.58 (s, 3H, CH_3), 6.95 (dd, 1H, Ar5H), 7.73 (dd, 1H, Ar4H), 8.32 (dd, 1H, Ar6H), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz (TMS/CCl_4).
13l - m. p. (uncorr) 29-31°C; IR (melt) 3040 cm^{-1} (Ar-H), 2910 (CH_2-H); NMR: δ 2.42 (s, 3H, CH_3); 6.92 (d, 1H, Ar3H), 7.57 (dd, 1H, Ar4H), 8.43 (d, 1H, Ar6H) $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/CCl_4).

3-Amino-2-picoline (20) and 5-Amino-2-picoline (21)

The method of van der Does[37] was employed to prepare 3-amino-2-picoline (20) and 5-amino-2-picoline (21). Three 210 ml stainless steel bombs were each filled with 100 ml of 28% aqueous NH_4OH, 2.8 g (11 mmol) of copper sulfate pentahydrate, and 15 g (87 mol) of the mixture of bromopicolines 13c and 13l. The combined reactions produced 27 g (96%) of a brown oil which was carried on to the next step without further purification.
20 and 21 - brown oil; IR (film): 3320 and 3200 cm^{-1} broad (NH-H).

3-Chloro-2-picoline (13d) and 5-Chloro-2-picoline (13e)

Following the method of Talik et al.[38], 1.0 g (9.3 mmol) of the aminopicoline mixture afforded 0.84 g (71%) of a clear, colorless oil consisting of 13d and 13e after steam distillation and without vacuum distillation. A pure sample of 13d was obtained through an alternate synthetic route.

13d - clear oil; IR (film): 3100 cm^{-1} (Ar-H), 2980 (CH_2-H); NMR: δ 2.54 (s, 3H, CH_3, 6.97 (dd, 1H, Ar5H), 7.51 (dd, 1H, Ar4H), 8.38 (dd, 1H, Ar6H), $J_{4,5}$ = 8 Hz, $J_{4,6}$ = 2 Hz, $J_{5,6}$ = 5 Hz (TMS/CCl_4).

6-Amino-3-chloro-2-picoline (23)

The procedure of Kress et al.[43] was used to prepare 6-amino-3-chloro-2-picoline (23). Starting with 27.0 g (250 mmol) of 6-amino-2-picoline (22), 30.3 g (85%) of 23 as a yellow oil was isolated and was used without chromatographic purification.

3-Chloro-6-hydroxy-2-picoline (24)

A modification of the method of Baumgarten et al.[39] was employed in the synthesis of 3-chloro-6-hydroxy-2-picoline (24). A 5.0 g (35 mmol) portion of 23 yielded 1.3 g (26%) of 24 as a colorless amorphous solid.
24 - colorless solid; IR (KBr): 3480 cm^{-1} (OH), 1700 (C=O); Anal: C,H,N.

3-6-Dichloro-2-picoline (25)

The method of Baumgarten et al.[39] was used to prepare 3,6-dichloro-2-picoline (25). A mixture of 1.3 g (9.1 mmol) of phosphorous pentachloride was heated under reflux at 110-115° C in an oil bath for 3 hours. After cooling, 0.26 ml (2.8 mmol) of phosphorous oxychloride and 0.25 g (1.2 mmol) of phosphorous pentachloride were added and the mixture was refluxed for an additional hour. After cooling, the mixture was neutralized with saturated sodium carbonate and extracted with 50 ml of methylene chloride. The methylene chloride was evaporated and the residue was loaded onto a short silica gel column (25 g). After elution with ethyl acetate, the solvent was evaporated, leaving 0.96 g (64%) of 25 as a brown oil.
25 - brown oil; IR (film): 3100 cm^{-1} (Ar-H), 2980 (CH_2-H); NMR: δ 2.57 (s, 3H, CH_3), 7.12 (d, 1H, Ar5H), 7.58 (d, 1H, Ar4H), $J_{4,5}$ = 9 Hz (TMS/$CDCl_3$); MS: m/e 160 (M^+-H), 126 (M^+-Cl).

3-Chloro-2-picoline (13d)

A modification of the procedure of Baumgarten et al.[39] was employed to prepare 3-chloro-2-picoline (13d). A 0.50 g (3.1 mmol) portion of 25 was reacted to give 0.16 g (41%) of a lightly colored oil. The modification of the Baumgarten et al.[39] procedure included neutralization with saturated aqueous sodium carbonate instead of sodium hydroxide, extraction with ethyl ether instead of benzene, and exclusion of the charcoal step. Physical and spectral data are reported in syntheses of 13d and 13e.

3-Iodo-2-picoline (13m) and 5-Iodo-2-picoline (13n)

A modification of the method of Riley and Perham was employed.[44] A 265 g (1.04 mol) portion of freshly ground iodine was dissolved in 485 ml of 30% oleum over 1 hour. The dropwise addition of 100 g (1.07 mol) of 2-picoline to the reaction mixture via addition funnel proceeded over 1 hour. The reaction was brought to 150°C for 1.5 hours during which time a vigorous evolution of gas occurred. The reaction was cooled to 120°C and

maintained for 12 hours. The mixture was reheated to 150°C for 3 additional hours, then cooled to room temperature. The reaction was poured over 1800 ml of ice and neutralized with sodium carbonate. The neutral aqueous mixture was extracted with 2 x 500 ml of ethyl acetate. The organic extracts were washed twice with brine, dried over sodium sulfate, filtered and flashed to a black oil. A vacuum distillation, b.p. 82-100°C at 5 mm (rpt b.p. 108-109, 18 mm, 17,[44] gave 62.3 g (27%) of 13n and 13m as a yellowish liquid. The roughly equimolar mixture could be partially resolved by LC (SG 70-230 mesh, 10% ethyl acetate/methylene chloride v/v, load ratio 1:100) and TLC (SG, ethyl ether) Rf 0.57 (13n) and 0.67 (13m).

13n yellow oil; NMR: δ 2.70 (s, 3H, CH_3), 6.75 (dd, 1H, Ar5H), 7.88 (dd, 1H, Ar4H), 8.38 (dd, 1H, Ar6H), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/CCl_4); MS: m/e 219 (M^+), 92 (M^+-I), 66 (M^+-CN,I), 51 (M^+-CH_3, CN,I).

13m yellow-green crystals, mp (corr) 46-48°C; NMR: δ2.44 (s, 3H, CH_3), 6.88 (d, 1H, Ar3H), 7.78 (dd, 1H, Ar4H), 8.62 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/CCl_4); MS: m/e 219 (M^+), 92 (M^+-I), 65 (M^+-HCN,I), 50 (M^+-CH_3, HCN,I).

3-Bromo-2-picolinealdehyde (14c) and 5-Bromo-2-picolinealdehyde (14m)

Employing a modification of the method of Markovac et al.[11], 10.1 g (58 mmol) of unresolved 13c and 13m was mixed with 14.8 g of freshly ground iodine. The resulting complex was warmed until homogeneity was achieved and after cooling, the solidified complex was dissolved in 58 ml of DMSO. The dark solution was then stirred in a 150-160° C oil bath for 40 minutes, during which time evolving dimethyl sulfide was distilled away from the reaction through a distillation head and condenser. After cooling, the pH of the reaction was adjusted to 6-7 with a saturated sodium carbonate solution. The mixture was then exhaustively extracted with ethyl ether. The ether extracts were washed with brine, dried with sodium sulfate, filtered and flashed. The residue was then purified by column chromatography (silica gel, loaded in methylene chloride and eluted with 10% v/v ethyl acetate/methylene chloride) to afford 1.1 g (10%) 14c, 2.2 g (20%) 14m and 3.2 g (30%) of the mixture.

14c - yellow needles, m.p. (corr) 64-65°C; IR (KBr): 3040 cm^{-1} (Ar-H), 2810 (CHO), 1720 (C=O); NMR: δ 7.50 (dd, 1H, Ar5H), 8.15 (dd, 1H, Ar4H), 8.75 (dd, 1H, Ar6H), 10.03 (s, 1H, CHO), $J_{4,5}$ = 9 Hz, $J_{4,6}$ = 2 Hz, $J_{5,6}$ = 5 Hz (TMS/CCl_4/d6-acetone); MS: m/e 187, (M^+), 159, 157 (M^+-CO), 78 (M^+-Br, CO); Anal: C, H, N.

14m - yellow needles, m.p. (corr) 90-93° C; IR (KBr): 3050 cm^{-1} (Ar-H), 2850 (CHO), 1700 (C=O); NMR: δ 7.75 (d, 1H, Ar3H), 7.97 (dd, 1H, Ar4H), 8.78 (d, 1H, Ar6H), 9.92 (s, 1H, CHO), $J_{3,4}$ = 9 Hz, $J_{4,6}$ = 2 Hz (TMS/CCl_4); MS; m/e 187, 185 (M^+), 159, 157 (M^+-CO), 78 (M^+-Br, CO); Anal: C, H, N.

3-Chloro-2-picolinealdehyde (14d) and 5-Chloro-2-picolinealdehyde (14e)

Preparation of 14d and 14e was as described for 14c and 14m. A 4.3 g (33.7 mmol) portion of 13d and 13e yielded, after chromatography, 0.67 g (14%) of 14d, 1.1 g (24%) of 14e and 0.95 g (20%) of the mixture.

14d - colorless needles, m.p. (uncorr) 45-47° C; IR (film): 2900 cm^{-1} (CHO), 1700 cm^{-1} (C=O); NMR: δ 7.48 (dd, 1H, Ar5H), 7.89 (dd, 1H, Ar4H), 8.73 (dd, 1H, Ar6H), 10.26 (s, 1H, CHO) (TMS/d_6-DMSO/$CHCl_3$); MS: m/e 141 (M^+), 113 (M^+-CO), 78 (M^+-CO,Cl); Anal: C, H, N.
14e - yellow solid; m.p. 58-61° C, literature[45] m.p. 60-62° C; IR (film): 2900 cm^{-1} (CHO), 1740 (C=O); NMR: δ 7.91 (m, 2H, Ar3, 4H), 8.79 (d, 1H, Ar6H), 10.09 (s, 1H, CHO) (TMS/$CDCl_3$).

3-Iodo-2-picolinealdehyde (14m) and 5-Iodo-2-picolinealdehyde (14n)

Employing a modification of the method of Markovac et al.[11], 11.9 g (54 mmol) of purified 13m and 13n was mixed with 13.3 g (52 mmol) of iodine. A solid picoline-iodine complex formed, which was gently melted, stirred to homogeneity and allowed to resolidify. The cake was broken up and dissolved in 25 ml of DMSO. The solution was added dropwise via an addition funnel to 30 ml of DMSO, which had been preheated to 150°C.The reaction mixture was maintained at 150-160°C for 40 minutes, during which time evolving dimethylsulfide was trapped in a 20% perchloric acid bubbler. The reaction was cooled to room temperature, neutralized with a saturated sodium bicarbonate solution and extracted exhaustively with ethyl ether. The combined ether extracts were washed with brine, dried over sodium sulfate, filtered and flashed to 13 g of a viscous black tar. The viscous tar was extracted with several portions of hexane. The hexane extracts were concentrated and loaded onto a silica gel column (70-230 mesh, 1:100 load ratio) and eluted with 10% ethyl acetate/methylene chloride (10-12 ml/min). The overall yield was 8.7 g (69%), of which 3.2 g (25%) was pure 5-iodo isomer 14n, 3.5 g (28%) pure 3-iodoaldehyde 14m and the remainder a mixture of isomers; TLC (SG, 10% ethyl acetate/methylene chloride v/v) Rf 0.48 (14m) and 0.65 (14n). Yields ranged from 30-69%. The individual isomers could be further purified by recrystallization from hexane.
14m - needles, pleasant minty odor, mp (corr) 67-69°C; IR: (KBr) 2860 cm^{-1} (CHO), 1700 (CO); NMR: δ 7.15 (dd, 1H, Ar5H), 8.32 (dd, 1H, Ar4H), 8.73 (dd, 1H, Ar6H), 9.83 (s, 1H, CHO), $J_{4,5}$=8 Hz, $J_{4,6}$=2 Hz, $J_{5,6}$=5 Hz (1% TMS/CCl_4); MS: m/e 233 (M^+), 205 (M^+-CO), 78 (M^+-CO, I); Anal. C,H,N.
14n - mp (corr) 112-113°C; IR: (KBr) 2840 cm^{-1} (CHO), 1695 (CO); NMR: δ 7.67 (d, 1H, Ar3H), 8.18 (dd, 1H, Ar4H), 8.98 (d, 1H, Ar6H), 9.96 (s, 1H, CHO), $J_{3,4}$=8Hz, $J_{4,6}$=2Hz (1% TMS/CCl_4); MS, m/e 233 (M^+), 205 (M^+-CO), 204 (M^+-CHO), 149 (M^+-CO,I, HCN); Anal. C,H,N.

5-Cyano-2-picolinealdehyde (14g)

Preparation of 14g was as described for the bromo series, 14c and 14m. A 15.0 g (127 mmol) portion of 5-cyano-2-picoline (13g) yielded, on evaporation of the ether extracts, 12.6 g (75%) of a dark black oil which was then dissolved in methylene chloride and enough ethyl acetate to afford solution. This solution was then loaded onto a short 120 g silica gel column and 14g was eluted with methylene chloride. Evaporation of the methylene chloride left 11.6 g (69%) of a yellow amorphous solid. Yields ranged from 49-69%.
14g - yellow amorphous solid, m.p. (uncorr) 65-85° C, sublimes at 55° C, 15 mm; IR (KBr): 2860 cm^{-1} (CHO), 2225 (C≡N), 1710 (C=O); NMR: δ 8.04 (m, 2H, Ar3, 4H), 9.01 (d, 1H, Ar6H), 10.07 (s, 1H, CHO), $J_{4,6}$ = 2 Hz

(TMS/$CDCl_3$); MS m/e 132 (M^+), 104 (M^+-CO), 103 (M^+-CHO), 77 (M^+-CO, HCN); Anal: C, H, N.

3-Iodo-2-pyridinealdehyde diethyl acetal (26)

Employing a modificaton of Bodor et al.[21], 1.22 g (5.2 mmol) of 3-iodo-picolinealdehyde 14m and 2.5 ml (15.0 mmol) triethylorthoformate were brought to a reflux under a nitrogen atmosphere in 10 ml of absolute ethanol saturated with HCl gas. The reaction was cooled to room temperature after 3-4 hours and nitrogen bubbled through the solution for 10-15 minutes to vent off excess HCl. The solution was transferred to a round-bottom flask and flashed to the ammonium salt. A 25 ml portion of saturated $NaHCO_3$ solution and 50 ml of ether were added to the residue and stirred. The ether layer was removed and washed with several small portions of water and with 2 X 15 ml of brine, dried over Na_2SO_4, filtered and flashed to a dark oil. The oil was loaded onto a short column of silical gel (70-230 mesh) and eluted with dry acetone. The resulting light tan oil was dried at 35-55 mm Hg for several hours, yielding 1.48 g (92% theoretical) of desired acetal 26.
26 - tan oil; NMR: δ 1.25 (t, 6H, $CH_2\underline{CH}_3$) 3.7 (1, 4H, $O\underline{CH}_2CH_3$) 5.75 (s, 1H, $\underline{CH}(OCH_2)_2$), 6.95 (dd, 1H, Ar5H), 8.15 (dd, 1H, Ar4H), 8.6 (dd, 1H, Ar6H), $J_{4,5}$ = 2Hz, $J_{4,6}$ = 2Hz, $J_{5,6}$ = 5Hz (TMS/CCl_4); MS: CI-NH_3, 308 (M^++1), 263 (M^++1-OCH_2CH_3).

3-Cyano-2-pyridinealdehyde diethylacetal (28)

Using the modified method of Friedman and Shecter[46], 1.4 g (4.6 mmol) of 26 and 0.41 g (4.6 mmol) of cuprous cyanide were brought to a vigorous reflux under a nitrogen atmosphere in 10 ml of dry dimethyl-formamide (DMF). The reaction went from colorless to deep red/brown over 6 hours. After cooling to room temperature, 80 ml of 20% aqueous sodium cyanide was added slowly with stirring to the reaction mix. The addition was exothermic as the copper complexes decomposed. The cocled mixture was extracted with several 20 ml portions of ether. Combined ether extracts were washed with 2 x 15 ml water and with 2 x 30 ml brine, dried over sodium sulfate, filtered and flashed to a deep red/brown oil. The oil was loaded onto a short silica gel column (25 g, 70-230 mesh) and eluted with 5% ethyl acetate/methylene chloride (v/v) to decolorize. The resulting light-tan oil, 0.42 g (44%), was single spot to TLC (SG, 5% ethyl acetate/methylene chloride).
30 - tan oil; IR: 2205 cm^{-1} (CN); NMR: δ 1.25 (t, 6H, $CH_3\underline{CH}_3$), 3.7 (dq, 4H, $O\underline{CH}_2CH_3$), 5.65 (s, 1H, $\underline{CH}(OCH_2)_2$), 7.35 (dd, 1H, Ar5H), 8.0 (dd, 1H, Ar4H), 8.75 (dd, 1H, Ar6H), $J_{3,4}$ = 8Hz, $J_{4,6}$ = 2Hz, $J_{4,5}$ = 5Hz (TMS/$CDCl_3$); MS: CI-NH_3, m/e 207 (M^++1), 161 (M^++1 - $HOCH_2CH_3$).

3-Methyl-2-pyridine carbaldoxime (15a)

The method of Markovac et al.[11] was followed in the preparation of 3-methyl-2-pyridine carbaldoxime (15a). A 20 g (190 mmol) portion of 2,3-lutidine (13a) afforded 5.8 g (23%) of 15a as a colorless solid.
15a - colorless amorphous soid, m.p. (corr) 151-155° C, literature[4] m.p. 152-154° C.

5-Methyl-2-pyridine carbaldoxime (15b)

Preparation of the intermediate 5-methyl-2-pyridinealdehyde (14b) was as described for the bromoseries, 14c and 14m. Starting with 20.0 g (187 mmol) of 2,5-lutidine (13b), 12.7 g (56%) of crude 14b was obtained from the ethereal extracts. The aldehyde 14b was then dissolved in 20-30 ml of methanol and 12.0 g (173 mmol) of hydroxylamine hydrochloride (prepared as as aqueous solution neutralized with solid sodium carbonate) was then added to the methanolic solution. The mixture was then heated to boiling for 5-10 minutes and allowed to stand overnight. The solution was then concentrated on a hot plate and after cooling was extracted three times with 100 ml portions of ether. After concentration of the ether extracts, 15b was precipitated from solution with cyclohexane. Filtration yielded 2.4 g (9%) of 15b as an amorphous yellow solid.
15b - yellow solid, m.p. (uncorr) 156-158°C literature[11] m.p. 158-159°C.

3-Bromo-2-pyridine carbaldoxime (15c)

A saturated aqueous solution of 0.20 g (2.9 mmol) of hydroxylamine hydrochloride was neutralized with sodium carbonate and added to a methanolic solution of 0.38 g (2.0 mmol) of aldehyde 14c. The solution was warmed gently to boiling and allowed to cool with stirring overnight. The product was collected by suction filtration, the supernatant concentrated and a second crop of product collected. Recrystallization from ethanol afforded 0.37 g (90%) of 15c.
15c - tan needles, m.p. (corr) 231-234° C: IR: 3400 (OH), 3130-2760 (oxime); NMR: δ 7.32 (dd, 1H, Ar5H), 8.10 (dd, 1H, Ar4H), 8.42 (s, 1H, CHON), 8.61 (dd, 1H, Ar6H), 11.88 (s, 1H, NOH), $J_{4,5}$ = 8 Hz, $J_{4,6}$ = 2Hz, $J_{5,6}$ = 5Hz (TMS/d_6-DMSO/d_6-Acetone); MS: m/e 202, 200 (M^+), 158, 156 (M^+-CHNOH); Anal: C,H,N.

5-Bromo-2-pyridine carbaldoxime (151)

Preparation of 151 was as described for 15c. A 0.41 g (2.2 mmol) portion of 141 was reacted with 0.25 g (3.6 mmol) of hydroxylamine hydrochloride to give 0.42 g (95%) of desired oxime 151.
151 - tan needles, m.p. (corr) 209-211°C; IR: 3400 cm^{-1} (OH), 3100-2790 (oxime); NMR: δ 7.73 (d, 1H, Ar3H), 7.97 (dd, 1H, Ar4H), 8.03 (s, 1H, CHNO), 8.63 (d, 1H, Ar6H), 11.45 (s, 1H, NOH), $J_{3,4}$ = 9 Hz, $J_{4,6}$ = 2 Hz (TMS/d_6-DMSO/$d_6$acetone); MS: m/e 202/300 (M^+), 158, 156 (M^+-CHNOH); Anal. C,H,N.

3-Chloro-2-pyridine carbaldoxime (15d)

Preparation of 15d was as described for 3-bromo-2-pyridinealdoxime (15c). A 0.84 g (5.9 mmol) portion of 14d reacted to yield 0.45 g (48%) of light-tan needles after standing 30 hours.
15d- light-tan needles, m.p. (corr) 213-215° C: IR (KBr): 1620 cm^{-1} (C=N); NMR: δ 7.40 (dd, 1H, Ar5H). 7.94 (dd, 1H, Ar4H), 8.57 (m, 2H, Ar6H and CHNOH), $J_{4,5}$ = 8Hz, $J_{4,6}$ = 2Hz, $J_{5,6}$ = 5Hz (TMS/d_6-DMSO/d_4-MeOH); MS: m/e 156 (M^+-H_2O), 121 (M^+-Cl), 103 (M^+-Cl, H_2O), 91 (M^+-Cl, NO); Anal: C,H,N.

5-Chloro-2-pyridine carbaldoxime (15e)

Preparation of 15e was as described for 15c. The reaction of 1.1 g (8.0 mmol) of 14e with 0.75 g (11.0 mmol) of hydroxylamine hydrochloride yielded 0.87 g (70%) of light-tan needles.
15e - light tan needles, m.p. (corr) 194-196° C, literature[38] m.p. 194-195°C;MS m/e 156 (M^+), 139 (M^+-OH), 126 (M^+-NO), 113 (M^+-CHNO).

3-Cyano-2-pyridine carbaldoxime (15f)

To a solution of 1.0 g (5.0 mmol) of 28 in 10 ml of acetone was added 5 ml of 2 N hydrochloric acid. This solution was refluxed for 1/2 hour and was then evaporated to about 1 ml. An aqueous solution of 0.34 g (4.9 mmol) of hydroxylamine hydrochloride was added to the concentrated solution. Precipitation of a tan granular solid occurred after stirring overnight. The yield of 15f was 71% (0.51 g).
15f - tan granular solid, m.p. (corr) 196-197° C (dec); IR (KBr): 2200 cm^{-1} (C≡N), 1620 (C=N); NMR δ 7.52 (dd, 1H, Ar5H), 8.18 (m, 2H, Ar4H and CHNOH), 8.71 (dd, 1H, Ar6H), 10.19 (s, 1H, CHNOH), $J_{4,5}$ = 9 Hz, $J_{4,6}$ = 2 Hz, $J_{5,6}$ = 5 Hz (TMS/d_6-DMSO/d_6-acetone).

5-Cyano-2-pyridine carbaldoxime (15g)

Preparation of 15g was as described for 15c. A filtered solution of 10.4 g (78.8 mmol) of 14g in methanol reacted with 6.12 g (88.1 mmol) of hydroxylamine hydrochloride to give 7.7 g (66%) of a yellow amorphous solid. Further precipitation was accomplished by adding 100 ml of water to the supernatant. Another 1.5 g (13%) of material was obtained in this manner. Yields ranged from 65-79%.
15g - yellow amorphous solid, m.p. (corr) 221-224° C; IR (KBr): 2200 cm^{-1} (C≡N), 1600 (C=N); NMR: δ 8.10 (m, 3H, Ar3, 4H and CHNOH), 8.93 (d, 1H, Ar6H), 11.81 (s, 1H, CHNOH) (TMS/d_6 DMSO/d_6-$CDCl_3$); MS: m/e 147 (M^+), 129 (M^+-H_2O), 117 (M^+-NO), 104 (M^+-CHNOH), 90 (M^+-NO,HCN), 76 (M^+-HCN, CHNOH); Anal: C,H,N.

Ethyl-2-carbaldoxime nicotinate (15h)

Preparation of the intermediate aldehyde was as described for 14c and 14m. Starting with 15 g (91 mmol) of ethyl-2-methyl nicotinate, 8.6 g (53%, 48 mmol) of the aldehyde was isolated. A solution of 3.3 g (47 mmol) of hydroxylamine hydrochloride dissolved in minimum water was added to a slightly less than saturated methanolic solution of the aldehyde (8.6 g, 48 mmol). Solid sodium carbonate was slowly added to bring the pH up to 6-7, at which point a gas evolved. The solution was allowed to stand overnight and the resulting precipitate was suction-filtered, yielding 3.8 g (22%) of a granular, yellowish solid.
15h - yellowish solid, m.p. (corr) 172-174° C; IR (KBr): 3420 cm^{-1} (OH), 1720 (C=O); NMR: δ 1.33 (t, 3H, CH_2CH_3), 4.32 (q, 2H, CH_2CH_3), 7.45 (dd, 1H, Ar5H), 8.06 (dd, 1H, Ar4H), 8.54 (s, 1H, CHNOH), 8.71 (dd, 1H, Ar6H), 11.71 (s, 1H, CHNOH), $J_{4,5}$ = 8 Hz, $J_{4,6}$ = 2 Hz, $J_{5,6}$ = 5 Hz (TMS/d_6-DMSO/d_6-acetone); MS: m/e 194 (M^+), 149 (M^+-OCH_2CH_3), 131 (M^+-OCH_2CH_3, H_2O), 78 (M^+-$CO_2CH_2CH_3$, CHNO); Anal: C, H, N.

Methyl-6-carbaldoxime nicotinate (15i)

Compound 15i was prepared by combining the procedures for the bromoaldehydes, 14c and 14m, and 3-bromo-oxime 15c. An 8.0 g (56 mmol) portion of methyl-6-methylnicotinate yielded 3.9 g (39%) of a yellow solid.
15i - yellow granular solid, m.p. (corr) 187-190° C, literature[11] m.p. 191-192° C.

3-Carboxamido-2-pyridine carbaldoxime (15j)

The basic method of Markovac et al.[11] was followed in the preparation of 3-carboxamido-2-pyridine carbaldoxime (15j). Into 30 ml of 28% aqueous ammonium hydroxide was suspended 1.00 g (5.2 mmol) of 15h. The slurry was stirred and warmed to 40-50° C until the solid dissolved. The solvent was evaporated to dryness, yielding 0.85 g (quant) of a colorless solid. Recrystallization, sublimation or chromatography did not appreciably purify this compound.
15j - colorless solid, m.p. (corr) 159-166° C (dec); IR (KBr): 1610 cm^{-1} ($CONH_2$), NMR; δ 7.51 (dd, 1H, Ar5H), 8.01 (dd, 1H, Ar4H), 8.64 (m, 2H, Ar6H and CHNOH), $J_{4,5}$ = 8 Hz, $J_{4,6}$ = 2 Hz, $J_{5,6}$ = 5 Hz (TSP/D_2O); MS: m/e 166 (M^++H), 148 (M^+-H_2O), 131 (M^+-H_2O, NH_3), 105 (M^+-H_2O, NH_3, CN), 91 (M^+-CONH_3, NO), 77 (M^+-CONH_3, CHNOH).

5-Carboxamido-2-pyridine carbaldoxime (15k)

Using the basic procedure of Markovac et al.[11], 3.5 g (19 mmol) of 15i was suspended in 100 ml of 28% aqueous ammonium hydroxide. The mixture was allowed to stir overnight, during which time the starting material dissolved. The solution was then evaporated to dryness and the residue was recrystallized out of ethanol/water with Norit decolorizing. The orange plates were suction-filtered to yield 2.4 g (75%).
15k - orange plates, m.p. (uncorr) 232-234° C, literature[11] m.p. 234-236° C.

3-Iodo-2-pyridine carbaldoxime (15m)

A saturated aqueous solution of 0.015 g (0.21 mmol) hydroxylamine hydrochloride was neutralized with sodium carbonate and added to a methanolic solution of 0.021 g (0.09 mmol) of aldehyde 14m. Within a short time a fine precipitate was evident. The solution was warmed gently to boiling and allowed to cool with stirring overnight. The white needles were collected by suction filtration, the supernate concentrated and a second crop of product collected. The combined crops afforded 0.019 g (82%) of oxime 15m, which was recrystallized from ethanol. Yields ranged from 42-82%.
15m - mp 235.5-238.5 (corr); IR: (KBr) 3400 cm^{-1} (brd, OH), 1610 (CH=N); NMR: δ 7.15 (dd, 1H, Ar5H), 8.34 (dd, 1H, Ar4H), 8.61 (m, 2H, Ar6H and CHNOH), $J_{4,5}$=8Hz, $J_{4,6}$=2Hz, $J_{5,6}$=5Hz (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 248 (M^+), 205 (M^+-CHNO), 204 (M^+-CHNOH), 104 (M^+-I, OH), 91 (M^+-NO, I); Anal. C,H,N.

5-Iodo-2-pyridine carbaldoxime (15n)

Preparation of 15n was as described for 15m. A 0.73 g (3.1 mmol) portion of 14n was reacted with 0.65 g (9.3 mmol) of hydroxylamine hydrochloride to give 0.69 g (89%) of 15n, which could be recrystallized from absolute ethanol.
15n - colorless needles mp (corr) 211-214°C; IR: (KBr) 3400 cm^{-1} (brd, OH), 1560 (CH=N); NMR: δ 7.64 (d, 1H, Ar3H), 8.00 (dd, 1H, Ar4H), 8.12 (s, 1H, CHNOH), 8.79 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/$CDCl_3$/CD_3SOCD_3); MS: m/e 248 (M^+), 204 (M^+-CHNOH), 121 (M^+-I), 104 (M^+-I,OH), 94 (M^+-I,HCN), 91 (M^+-I,NO); Anal. C,H,N.

3-Methyl-2-pyridine carbaldoxime methiodide (16a)

A modification of the method of Poziomek et al.[47] was used to prepare 3-methyl-2-pyridine carbaldoxime methiodide (16a). A 5.0 g (37 mmol) portion of 15a was refluxed with 9.0 ml (150 mmol) of methyl iodide in 70 ml of methyl ethyl ketone for 18 hours yielding 6.1 g (60%) of bright yellow needles on suction filtration.
16a - bright yellow needles, m.p. (uncorr) 202-203° C (dec), literature[4] m.p. 201° C; IR (KBr): 1610 cm^{-1} (C=N); NMR: δ 2.60 (s, 3H, Ar3CH_3), 4.37 (2, 3H, N-CH_3), 7.99 (dd, 1H, Ar5H), 8.65 (m, 3H, Ar4, 6H and CHNOH)(D_2O).

5-Methyl-2-pyridine carbaldoxime methiodide (16b)

The preparation of 16b was as described for 3-methyl-2-pyridine carbaldoxime methiodide (16a). A 2.0 g portion of 15b with 4.5 ml of methyl iodide in 100 ml of methyl ethyl ketone yielded 2.4 g (70%) of yellow needles.
16b - m.p. (corr) 145° C, literature[11] m.p. 155-156° C.

5-Chloro-2-pyridine carbaldoxime methiodide (16e)

A modification of the method of Poziomek et al.[47] was employed to prepare 5-chloro-2-pyridine carbaldoxime methiodide (16e). A 0.50 g (3.5 mmol) portion 15e, 10 ml of methyl ethyl ketone and 5.5 ml (88 mmol) of methyl iodide were combined into a pressure vessel. The head space of the vial was thoroughly purged with nitrogen. After sealing the vessel, it was placed in an 80° C oil bath for 42 hours. The bright yellow precipitate suction filtered upon cooling gave a yield of 0.55 g (58%).
16e - bright yellow solid; m.p. (corr) 205-208° C (dec.), literature[11] 203-204° C; IR (KBr): 1650 cm^{-1} (C=N); NMR: δ 4.41 (s, 3H, CH_3), 8.47 (m, 2H, Ar3H, 4H), 8.70 (s, 1H, CHNOH), 9.09 (s, 1H, Ar6H) (D_2O/d_6-DMSO).

5-Carboxamido-2-pyridine carbaldoxime methiodide (16k)

Preparation of 16k was as described for 16e. Starting with 2.00 g (12.1 mmol) of 15k and 11.5 ml (185 mmol) of methyl iodide in 100 ml of methyl ethyl ketone, a yield of 82% (3.06 g) of yellow needles was afforded. Yields ranged from 70-82%.
16k - yellow needles, m.p. (corr) 178-197° C, literature[11] m.p. 169-170° C; IR (KBr): 1680 cm^{-1} (C=O), 1620 (C=N); NMR: δ 4.50 (s, 3H, CH_3), 8.50 (d, 1H, Ar3H), 8.71 (s, 1H, CHNOH), 8.82 (dd, 1H, Ar4H), 9.25 (d, 1H, Ar6H) (TSP/D_2O).

3-Iodo-2-pyridine carbaldoxime methiodide (16m)

A modification of the procedure of Poziomek, Hackley and Steinberg[47] was used. A 1.60 g (6.45 mmol) portion of oxime 15m was dissolved in approximately 100 ml of methylethylketone in a 250 ml pressure bottle. Immediately after purging the head space with nitrogen, 4.0 ml (0.064 mol) methyliodide was added and the vessel sealed with a Teflon plug. The vessel was heated in an oil bath at 50°C for 24 hours and cooled to room temperature, and the product was suction-filtered and washed. The yellow-orange needles, 1.92 g (76%), required no further purification.

16m - yellow-orange needles, mp (corr) 195-205°C (dec); IR: (KBr) 3200 cm^{-1} (brd OH), 1620 (CH=N); NMR: δ 4.31 (s, 3H, CH_3), 7.68 (dd, 1H, Ar5H), 8.41 (s, 1H, CHNOH), 8.83 (d, 1H, Ar6H), 8.96 (d, 1H, Ar4H), $J_{4,5}$=8Hz, $J_{5,6}$=6Hz (D_2O/D_6-DMSO with H_2O reference).

5-Iodo-2-pyridine carbaldoxime methiodide (16n)

Preparation of 16n was as described for 16m. Starting with 4.61 g (0.0:86 mol) of 15m and 34 ml (0.55 mol) of methyl iodide, the reaction was run in 150 ml absolute ethanol for 3 days at 75°C. A bright-yellow crystalline solid 16n, 5.70 g (78%), was isolated. A second crop was obtained by concentrating filtrate and adding ethyl acetate.

16m - mp (corr) 220-223°C (dec); IR: (KBr) 400 cm^{-1} (brd OH), 3100-2730 (oxime), 1620 (C=N); NMR: δ 4.37 (s, 3H, CH_3), 8.18 (d, 1H, Ar3H), 8.67 (s, 1H, CHNO), 8.89 (dd, 1H, Ar4H), 9.20 (d, 1H, Ar6H), $J_{3,4}$=9H, $J_{4,6}$=2Hz (D_2O/D_6-DMSO, H_2O reference); Anal. C,H,N.

3-Iodo-2-pyridine carbaldoxime methochloride (17m)

The halide exchange employed was the method of Kondritzer et al.[48] Dowex-1 resin (chloride form) was rinsed with methanol and repeatedly with deionized water. The final water rinse was tested with 1% silver nitrate solution to ensure that it was free of halogens. A 4.7 g (12 mmol) portion of 16m as the iodide salt was stirred for 4 hours with 150 g of resin in a minimum amount of water. The suspension was filtered and the filtrate flashed to a residue. The resulting chloride salt was recrystallized from ethanol/ethyl acetate to afford 2.5 g (69%) of colorless crystals.

17m (chloride form) mp 198-202°C (dec); Anal. C,H,N.

5-Iodo-2-pyridine carbaldoxime methochloride (17n)

Preparation of 17n (chloride form) was as described for 17m above. Starting with a 5.5 g (14 mmol) portion of 17n (iodide form), a glassy residue of the desired product was obtained. The salt could be recrystallized from either ethanol/ethyl acetate to afford 3.05 g (72%) of material or water to give 2.70 g (64%) of 5a.

17n (chloride form) mp (corr) 193-195°C (dec); Anal. $C_7H_8N_2OClI \cdot 1/2$ CH_3CH_2OH calc. C,29.88 H,3.44 N,8.71; fnd C,29.44 H,3.52 N,8.57; mp (corr) 198-200°C (dec); Anal $C_7H_8N_2OClI \cdot 1/2$ H_2O C,H,N.

3-Methyl-2-pyridine carbaldoxime methochloride (17a)

The basic procedure of Kondritzer et al.[48] was used. A Dowex-1X8 resin was prepared as the choride form by first washing with methanol and then stirring 15 minutes with each of three portions of 1 N sodium chloride. The resin was then washed with water until the washings no longer gave a positive chloride test with 1% silver nitrate. The resin was then allowed to swell in water overnight. A solution of 1.0 g (3.6 mmol) of 16a in minimum water was stirred for 3 hours with 30 g (wet weight, 30 meq) of the resin. The resin was filtered off and extracted three times with water. The water extracts and filtrate were then flashed to dryness and the residue was recrystallized out of ethanol. Crystallization was initiated with the addition of ethyl acetate. The tan needles were filtered to yield 0.48 g (72%).
17a - tan needles, m.p. (corr) 205-208° C (dec); spectra were identical to 16a; Anal: C, H, N.

5-Methyl-2-pyridine carbaldoxime methochloride (17b)

Preparation of 17b was as for 17a above. A yield of 0.38 g (57%) of 17b was obtained from 1.0 g of 16b.
17b - tan needles, m.p. (corr) 196-199° C (dec.); spectra identical to 16b; Anal: (+ 1-H_2O): C, H, N.

3-Bromo-2-pyridine carbaldoxime methochloride (17c)

Using the method of Bell and Zalay[49], 3.0 ml of methyl toluenesulfonate was added to 0.50 g (2.5 mmol) of 15c. The slurry was heated with stirring in a 120-125° C oil bath just until the oxime totally dissolved; about 45 minutes. The solution was cooled and dissolved into about 25 ml of anhydrous methanol. The product was precipitated with anhydrous ether and the supernatant was decanted 4 hours later from a gummy, greenish precipitate. The precipitated was redissolved in anhydrous methanol, which was then added to a 15-20 ml methanolic slurry of Dowex-1 resin (prepared as the chloride form, as described for 17a and extensively washed with anhydrous methanol to exclude water). After stirring for 3 hours, the resin was filtered off and the methanol was flashed to a residue. Recrystallization of the residue from ethanol/ethyl acetate, as for 17a, yielded 0.19 g (30%) of 17c.
17c - green crystals, m.p. (corr) 202-204° C (dec); IR (KBr): 3480 cm^{-1} broad (OH), 1620 (C=N); NMR: δ 4.44 (s, 3H, CH_3), 7.94 (dd, 1H, Ar5H), 8.54 (s, 1H, CHNOH), 8.88 (m, 2H, Ar4, 6H) (D_2O); Anal: C, H, N.

5-Bromo-2-pyridine carbaldoxime methiodide (161)

Preparation of 161 was as described for 16m. Starting with 0.46 g (2.3 mmol) of 151 and 3.5 ml (5.6 mmol) of methyl iodide, the reaction was run in 20 ml of methylethylketone for 24 hours at 90°C. A bright-yellow crystalline solid, 161, 0.35 g (45%), was isolated. The use of ethyl acetate as a reaction solvent results in yields up to 72%.
161 - yellow needles, m.p. (uncorr) 213-215°C; IR: (KBr) 3400 cm^{-1} (OH), 3100-2710 (oxime); NMR: δ 4.53 (s, 3H, CH_3), 8.39 (d, 1H, Ar3H), 8.68 (s, 1H, CHNO), 8.82 (dd, 1H, Ar4H), 9.58 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TMS/d6-DMSO/ d6-acetone).

5-Bromo-2-pyridine carbaldoxime methochloride (171)

Preparation of 171 (chloride form) was as described for 17m. Starting with a 0.35 g (1 mmol) portion of the methiodide salt, 0.18 g (70%) of the desired methochloride salt was isolated as colorless needles.
171 - (chloride form) m.p. 199-201°C; IR: (KBr) 3400 cm^{-1} (OH), 3100-2710 (oxime); NMR: δ 4.40 (s, 3H, CH_3), 8.33 (d, 1H, Ar3H), 8.68 (s, 1H, CHNO), 8.72 (dd, 1H, Ar4H), 9.12 (d, 1H, Ar6H), $J_{3,4}$=9Hz, $J_{4,6}$=2Hz (TSP/D_2O).

3-Chloro-2-pyridine carbaldoxime methochloride (17d)

Preparation of 17d was as described for 17c. A 0.20 g (1.3 mmol) portion of 15d reacted to yield 0.010 g (4%) of colorless crystals.
17d - colorless crystals, m.p. (corr) 209-211° C (dec); IR (KBr): 3460 cm^{-1} broad (OH), 1640 (C=N); NMR: δ 4.43 (s, 3H, CH_3), 8.15 (dd, 1H, Ar5H), 8.75 (m, 3H, Ar4H, 6H and CHNOH) (TSP/D_2O); Anal: C, H, N.

5-Chloro-2-pyridine carbaldoxime methochloride (17e)

Preparation of 17e was as described for 17a. A 53% (0.20 g) yield of 17e was obtained from 0.55 g (1.8 mmol) of 16e.
17e - tan crystals, mp (corr) 205-208° C (dec); spectra were identical with that of 16e; Anal: C,H,N.

5-Cyano-2-pyridine carbaldoxime methochloride (17g)

Preparation of 17g was as described for 17c. Starting with 4.0 (27 mmol) of 15g, a dark black oil and crystalline mixture was obtained. This mixture was extracted quickly with two 10 ml portions of absolute ethanol to decolorize the product. The light brown solid residue was recrystallized out of ethanol/ethyl acetate as for 17a. The yield was 1.0 g (19%) of light brown crystals.
17a - brown flakes, m.p. (corr) 223-225° C (dec); IR (KBr): 2220 cm^{-1} (C=N), 1650 (C=N); NMR: δ 4.48 (s, 3H, CH_3), 8.78 (m, 3H, Ar3, 4H and CHNOH), 9.42 (d, 1H, Ar6H), $J_{4,6}$ = 2 Hz (D_2O); Anal: C, H, N.

5-Carboxamido-2-pyridine carbaldoxime methochloride (17k)

Preparation of 17k was as described for 17a. The iodide salt, 3.06 g (10 mmol) of 16k was converted to 1.46 g (68%) of tan flakes.
17k- tan flakes, m.p. (corr) 216-218° C (dec); spectra were identical with that of 16k; Anal: C, H, N.

N-Methyl-2-cyano-1,2,3,6-tetrahydropyridine-2-carbaldoxime (33)

The procedure of Bodor, Shek and Higuchi[21] was employed starting with 2.5 g (9.5 mmol) of 2-PAM (3), 0.53 g (38%) of 33 was isolated as a light tan solid. The material darkened considerably with time when stored at room temperature in air. Even when stored under nitrogen, the compound was not especially stable.
33 - mp 101-104°C (rpt 112-114, ref. 21); IR: (KBr) 3200 cm^{-1}(brd OH), 2300 (CN), 1650 (C=N); NMR: δ 2.38 (s, 3H, CH_3), 3.15 (m, 4H, CH_2CH), 5.75 (m, 2H, $CH_2CHCHCH_2$), 7.35 (s, 1H, CHNOH), (TMS/CD_3COCD_3).

N-Methyl-1,2,3,6-tetrahydropyridine-2-carbaldoxime-2-thiocyanate (34)

A modification of the method of Bodor, Shek and Higuchi[21] was used. Into 10 ml of water was dissolved 1.5 g (15.4 mmol) of potassium thiocyanate and 1.0 g (3.8 mmol) of 2-PAM (3). The solution was degassed with nitrogen and chilled to 0°C and the pH adjusted to 1-2 with concentrated hydrochloric acid. The aqueous solution was layered with 50 ml of ethyl ether and 0.2 g (5.3 mmol) of sodium borohydride was added in one portion. The temperature was maintained at 0°C until the ebullition ceased and then the reaction was allowed to warm to room temperature slowly. The nitrogen atmosphere was maintained and the pH monitored throughout the reaction. The pH leveled off at 7-8 and remained at that figure through most of the reaction. After 4 hours the aqueous layer was saturated with sodium chloride, the ether layer removed and the aqueous layer extracted with a second 50 ml portion of ether. The combined ether extracts were washed with brine, dried over sodium sulfate, filtered and evaporated to 0.240 g (32%) of a light pink solid, 34. The product darkened considerably with time even when stored under a nitrogen atmosphere.

34 - mp (corr) 94-96°C; IR (KBr) 2110 cm^{-1} (SCN), 1665 (HC=N); NMR: δ 2.35 (s, 3H, CH_3), 3.20 (m, 4H, $CH_2CHCHCH_2$), 5.70 (m, 2H, CH_2CHCH), 7.45 (d, 1H, CHNOH), (TMS/CD_3COCD_3).

N-Methyl-1,2,3,6-tetrahydropyridine-2-bromo-2-carbaldoxime (35)

Preparation of 35 was as described for 34 above. Starting with a 0.264 g (1.0 mmol) portion of 2-PAM (iodide salt form), 0.064 g (29%) of the desired bromine addition product was isolated.

35 - colorless solid, m.p. 96-99°C; IR (KBr) 1670 (C=N); NMR: δ 2.35 (s, 3H, CH_3), 3.20 (m, 4H, CH_2 $CHCHCH_2$), 5.72 (m, 2H, CH_2 CHCH), 7.30 (s, 1H, CHNO) (TMS/d_6-acetone).

% Conversion Determination - 33-35 to 2-PAM (3)

Samples of 33-35 (10^{-3} M) were prepared in 150 mM Na_3PO_4 buffer (pH range 6.5-8.5, adjusted with H_3PO_4 or Na_3PO_4). Samples were stirred in a vessel open to atmosphere and aliquots were withdrawn, mixed 1:1 with mobile phase and injected (20 µl on column) at 5, 10, 20, 40, 80, 100, 320 minutes and 24 hours. Identification and quantitation were performed via HPLC, using a reversed-phase C18 column (see Experimental Methods - 1. Equipment and Reagents) eluted with 20% acetonitrile/aqueous paired ion reagents (0.01 M heptane sulfonic acid, 0.001 tetraethylammonium perchlorate, adjusted to pH 3.5 with acetic acid) at 1.5 ml/minute. 2-PAM exhibited a t_R of 2.7-3 minutes.

3. Bioassay

a. Immobilized Acetylcholinesterase Assay

Purified AChE (E.C.3.1.1.7, Electrophorus electricus) and human RBC AChE were immobilized, using a modification[38] of the technique reported by Ngo, Laidler and Yam[40]. The modifications were as follows: Precision-ground, 4 mm spherical, low density polyethylene beads were

used; all reactions and washes were conducted using an overhead mechanical stirrer with Teflon paddle; treatment of the beads with thionyl chloride, ethylenediamine and required rinses was carried out under a nitrogen atmosphere; the beads were treated with 10% glutaraldehyde for 3 hours with one solution change; the attachment of glutaraldehyde to the bead could be monitored for completeness by incubating the beads in the presence of DTNB, since any noncapped amino groups on the bead surface reacted with DTNB resulting in the generation of a chromophore which was monitored at 412 nm; the enzyme was loaded onto the beads in a 2 hour room temperature incubation; and MOPS buffer (0.1M, pH 7.8) replaced phosphate buffer throughout.

Beads containing immobilized enzyme were stored at -16° C in MOPS buffer containing 40% glycerin. The stability of the enzyme activity was effected by freeze-thaw cycles; therefore, the beads were frozen in small batches. The activity was stable for up to 4 months when stored as described. Enzyme activity per bead averaged 0.5 units/bead; however, significant bead-to-bead variation was observed. Under normal assay conditions (0.1 M MOPS buffer, pH 7.8, 37° C), 1.5-2%/h loss of enzyme activity was observed.

b. Measurement of AChE Activity

The enzyme activity assay was based on the Ellman technique[28]. Acetylthiocholine (in 90% ethanol) and DTNB (in 95% ethanol) were added to 30 ml of MOPS buffer as above to give concentrations of 1×10^{-3} M and 5×10^{-4} M, respectively. Figure 12 is a schematic of the closed loop flow-through system employed in the assay. The peristaltic pump was routinely run at 5.8 ml/min and the column effluent monitored at 412 nm. The column was a polypropylene cylinder which contained immobilized enzyme beads packed with alternating glass beads which optimized surface area and flow characteristics. A solution in the flask containing substrate and chromogen was directed via switching valves through the column. The flask contents were cycled so that a stable baseline rate of enzyme activity was observed on the recorder trace. An excess of organophosphate (e.g., DFP, soman, paraoxon) was added and after all enzyme activity ceased (approximately 5 minutes), the system was flushed with fresh buffer via switching valves.

Reactivators were routinely prepared in 200 ml volumes; however, some of the highest concentrations of 2-PAM and MINA were prepared in 100 ml volumes to conserve material. As mentioned earlier, phosphorylated oximes are potent enzyme inhibitors. To reduce the possibility of the phosphorylated oximes inhibiting the reactivated enzyme, the eluent was passed out of the system for the first 5 minutes of exposure to the reactivators. A fresh solution of reactivator was then recycled for the remaining exposure time. We observed no difference between the above procedure and pumping reactivator through the column in a single-pass fashion for the entire 45 minute exposure time. In the case of 2-PAM, we also examined the concentrations of reactivator (295 nm) via HPLC, both entering and exiting the column, and found no difference.

The detector output was recorded as absorbance units vs time which was converted to rate of substrate hydrolysis using the conversion of Ellman[28].

$$\frac{\Delta Abs/min}{1.36 \times 10^4} = \text{mol thiocholine/l.min.}$$

The substrate concentration routinely used in the assay was at saturation level, and the flow rate was an intermediate value. There was no apparent effect of flow rate on the regeneration of DFP-inhibited immobilized enzyme.

c. In Vivo - Animal Survival Studies

Adult male mice (CFI outbred), 25-35 g, obtained from Sasco, Inc., Omaha, NE, were used. Experiments were performed, using the same shipment of animals for consistency. Freshly prepared DFP (6 mg/kg, 2 X LD_{50}) in water was injected s.c. (20-35 µl). Reactivators (up to 100 mg/kg) were prepared in water and injected into the left hind limb i.m. (100 µl, 1% body volume), except for pro-2-PAM (8), which was administered i.v. (tail, 20 second infusion) in freshly prepared and deoxygenated citric acid buffer (50 mM, pH 3.5).[29] (For ease of use, i.m. injections were preferred. While i.m. and i.v. injections of 2-PAM did not significantly change biodistribution, pro-2-PAM had to be injected i.v. due to other studies.[29]) Groups of four or five animals/dose and four dose levels were used. Survivors were counted at 24 hours.

d. HPLC Assay for Pyridinium Oximes

The parent drug, 2-PAM, was injected i.m. (50 mg/kg in water). (There were no significant differences in the brain levels of the drug after i.m. or i.v. administration.) The pro-2-PAM (50 mg/kg in 0.05 M citrate buffer, pH 3.0) was injected iv in the lateral tail vein, unless otherwise noted.

Animals were decapitated at the end of the time period. Blood was collected with 0.15% EDTA to prevent clotting. The sample was immediately centrifuged for 5 minutes at 1000 x g. Perchloric acid was added to the plasma sample to precipitate proteins and centrifuged for 10 minutes (1000 x g). The clear supernatant was filtered through a nitrocellulose filter (0.45 µm), basified with NaOH and stored at -20° C until direct injection on the HPLC. One hundred µl of the packed RBC were diluted 5-fold with water and frozen. Perchloric acid was added to the thawed sample to precipitate proteins and the supernatant was centrifuged, filtered and basified as above.

Organs were surgically removed, weighed and homogenized in a rotary homogenizer (Tekmar Tissumizer) for 15-45 seconds in 1 ml of water, except for brain samples analyzed for unconverted pro-2-PAM (see below). Concentrated perchloric acid was added and the sample centrifuged for 10-15 minutes (1000 x g). The clear supernatant was filtered (0.45 µm), basified and stored as above until direct HPLC injection after equilibrating to room temperature.

It was noted that a strongly acidic medium stopped the conversion of pro-2-PAM to 2-PAM in the presence of oxygen or even a strong oxidant such as $AgNO_3$. Therefore, a sample could be analyzed with the unconverted pro-2-PAM still present. Also, it was found that the dissection of the brain and its exposure to the air seemed to increase the conversion process. Therefore, to analyze these brain samples for only the 2-PAM that was present in vivo at the moment of decapitation, the whole head was quickly immersed in a dry-ice bath of methylene chloride (-70° C). The brain was then dissected out while submerged in a strongly acidic buffer (0.3 M phosphate buffer, pH = 1.5). The tissue was weighed in the acid buffer and gently ground by hand with glass beads while in a sonication bath to avoid introducing excessive air. Perchloric acid was added and the sample was centrifuged and filtered as above. The samples were analyzed for pro-2-PAM immediately.

A PRP-1 reversed-phase column (22 cm x 4.6 mm + 3 cm x 4.6 mm guard column, 10 µm spherical beads, Brownlee Cartridge system) was used throughout this study. The unique solid phase in this column has a polymer backing which allows a wide range of pH systems (1-13). This allowed the detection of small amounts of 2-PAM under acidic or basic conditions. Standard silica-based, reversed-phase columns do not tolerate such a wide pH range (pH = 2 to 8). To analyze 2-PAM, a basic solvent system was used (0.1 M Na_2CO_3, pH = 10.5). Samples were detected at 334 nm by a Kratos SF769Z UV detector and quantified on a Spectra Physics 4270 integrator. 2-PAM eluted at 4.6 minutes with a flow rate of 1.0 ml/min from a Model 112 Beckman Pump. All analyses were done at room temperature for this study. Twenty µl injections were used throughout and the minimum detection was 10 ng on column.

In the acidic system (0.1 M phosphoric acid buffer, pH = 2.5), 2-PAM provided a retention time also of 4.6 minutes with a flow rate of 1.0 ml/min as above. The maximum wavelength of absorbance of 2-PAM in this buffer is 293 nm, but background interference from biological material necessitated changing the wavelength up to 312 nm to reduce this interference. The minimum detection at this wavelength was 17 ng on column.

Pro-2-PAM elutes at just over 6 minutes from injection. Analysis of pro-2-PAM from an in vivo sample directly at 251 nm showed poor resolution because of the great degree of biological background interference. Pro-2-PAM eluted on the shoulder of this material at this wavelength. By using the computing integrator, pro-2-PAM could be analyzed although with a small amount of variation. Pro-2-PAM did not appear in chromatograms at 312 nm in biological samples with the acidic system.

To study the conversion process of the prodrug in vitro, the supernatant of homogenized, centrifuged brains was inoculated with the given amount of pro-2-PAM. $AgNO_3$ was added as a solid to ensure complete oxidation for maximum conversion determination. After 10 minutes perchloric acid was added to precipitate proteins and the sample was centrifuged, basified with NaOH and filtered. The sample was analyzed on the HPLC for 2-PAM with the basic solvent system.

The conversion rate experiments were carried out in a 1:1, bovine serum (Pel Freeze Co.)-MOPS buffer (pH = 7.4) mixture at 37°C with an air stream passing over the surface continuously. An aliquot was withdrawn at the given time and added to a perchloric acid mixture, which precipitated the proteins and ceased conversion immediately. The samples were then analyzed on the acid solvent system at 293 nm. External standards were also mixed in the serum buffer to provide background correction.

To determine the LD_{50} of 2-PAM or pro-2-PAM, mice were injected s.c. (neck) with 2 X LD_{50} dose of DFP (6.0 mg/kg) and immediately the given dose of antidote was administered i.m. (2-PAM) or i.v. (pro-2-PAM as above). The dose was given immediately after the organophosphate in order to test the drug efficacy under near-clinical conditions rather than a prophylactic administration. Groups of 5 mice for each dose were used and the number of survivors at 24 hours was used to determine the ED_{50}.

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